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(54) Title: CHIMERIC RECEPTORS FOR JAK-STAT SIGNAL TRANSDUCTION

(57) Abstract

The invention provides a chimeric receptor for cellular activation comprising an extracellular domain capable of binding to a cognate molecule, and a cytokine intracellular domain capable of interacting with a Jak-Stat protein, thereby participating in signal transduction, with the proviso that the cytokine intracellular domain is not naturally associated with the extracellular domain. The present invention harnesses cytokine and growth factor receptor cellular activation pathways for responses to different receptor ligands, thus enabling reconstitution of cellular activation to a ligand, such as a cytokine or growth factor, where some deficiency prevents activation by the native pathway; targeting of effector cells to tumors, virally infected cells, or autoimmune cells; and creating new cytokine or growth factor sensitivities in responder cells, e.g., to develop an assay system in which cells not normally responsive to a given cytokine are rendered responsive. In specific embodiments, the cytokine intracellular domain interacts with a Jak protein selected from the group consisting of Jak1, Jak2, Jak3, and Tyk2. In another embodiment, preferably the cytokine intracellular domain interacts with a Stat protein selected from the group consisting of Stat1α, Stat1β, Stat2, Stat3, Stat4, Stat5 and Stat6. In specific examples, various functional chimeric constructs, including γR2/γR1, γR2/αR1, γR2/αR2, γR2/CRF, γR2/γC, IL-10R/γR1, EpoR/γR1, EpoR/γR2, γR1/EpoR, γR2/EpoR, H1/γR1, and L1/γR2, were prepared.

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CHIMERIC RECEPTORS FOR JAK-STAT SIGNAL TRANSDUCTION

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FIELD OF THE INVENTION

The present invention relates to genetically engineered chimeric receptor proteins, cells expressing the same, and uses thereof to overcome deficiencies in cellular activation mechanisms and to define new therapeutic modalities.

BACKGROUND OF THE INVENTION

Cytokine Activation of Cells

Each cytokine which utilizes the Jak-Stat signal transduction pathway activates a distinct combination of members of the Jak and Stat families. Thus, either the Jaks, the Stats or both could contribute to the specificity of ligand action. The Jak-Stat signal transduction pathway was first discovered for interferon alpha (IFN-α) and interferon gamma (IFN-γ) by the complementation of mutant cell lines defective in response to IFN-γ and/or IFN-α [Velazquez *et al.*, *Cell*, **70**:313-322 (1992); Watling *et al.*, *Nature*, **366**:230-235 (1993); Müller *et al.*, *Nature*, **366**:129-135 (1993) and *EMBO*, **12**:4221-4228 (1993); Darnell *et al.*, *Science*, **264**:1415-1421 (1994); Leung *et al.*, *Mol. Cell. Biol.*, **15**:1312-1317 (1995)]. It has subsequently been shown that the same general pathway is activated by most cytokines and some growth factors [for review, see Ihle and Kerr, *Trends Genet.*, **11**:69-74 (1995); Taniguchi, *Science*, **268**:251-255 (1995)]. This pathway is activated predominantly through receptors which do not possess intrinsic intracellular kinase domains and belong to the class I or class II cytokine receptor superfamily. The lack of inh rent catalytic activity in th se receptors is overcome through the use of r ceptor-associated kinases of the Janus kinase (Jak) family. Upon ligand binding, the receptor chains oligomerize, allowing the associated kinas s to interact and likely crossactivate each other by tyrosine

phosphorylation. Subsequ ntly, the activated Jaks directly phosphorylate the intracellular domains of the receptors on specific tyrosine residues. This phosphorylation allows the selective recruitment of SH2-domain containing proteins, particularly Stats (Signal transducers and activators of transcription), through a specific interaction between the Stat SH2 domains and the phosphotyrosines within the Stat recruitment sites of the intracellular domains of the receptor chains. These receptor-associated Stats are then rapidly phosphorylated, likely by the activated Jaks [Quelle *et al.*, *supra*]. The phosphorylation of the Stats is followed by Stat dimerization, translocation to the nucleus and activation of cytokine inducible genes.

The Jak and Stat families are growing rapidly. The Jak family consists of four members so far: Jak1, Jak2, Jak3 and Tyk2 [Wilks *et al.*, *Mol. Cell. Biol.*, **11**:2057-2065 (1991); Silvennoinen *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**:8429-8433 (1993); Firmbach-Kraft *et al.*, *Oncogene*, 5:1329-1336 (1990); Witthuhn *et al.*, *Cell*, **742**:27-236 (1994); Kawamura *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**:6374-6378 (1994); for review see Ziemiecki *et al.*, *Trends Cell Biol.*, **4**:207-212 (1994); Ihle *et al.*, *Trends in Biochemical Sciences*, **19**:222-227 (1994); Ihle *et al.*, *Trends Genet.*, **11**:69-74 (1995); Ihle and Kerr, *supra*]. The Stat family now includes seven different members, which have been cloned: Stat1α, Stat1β, and Stats2-6 [Schindler *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**:7836-7839 (1992); Fu *et al.*, *Proc. natl. Acad. Sci. USA*, **89**:7840-7843 (1992); Zhong *et al.*, *Science*, **264**:95-98 (1994) and *Proc. Natl. Acad. Sci. USA*, **91**:4806-4810 (1994); Yamamoto *et al.*, *Mol. Cell. Biol.*, **14**:4342-4349 (1994); Akira *et al.*, *Cell*, **77**:63-71 (1994); Wakao *et al.*, *Cell*, **70**:2182-2191 (1994); Hou *et al.*, *Science*, **265**:1701-1706 (1994); International Patent Publication WO 95/08629, by Darnell *et al.*, published March 30, 1995; and International Patent Publication No. WO 93/19179, by Darnell *et al.*, published September 30, 1993] and several others, which were identified by electrophoretic mobility shift assays, but have not been cloned yet [Meyer *et al.*, *J. Biol. Chem.*, **269**:4701-4704 (1994); Finbloom *et al.*, *Mol. Cell. Biol.*, **14**:2113-2118 (1994); Tian *et al.*, *Blood*, **84**:1760-1764 (1994); Finbloom and Winestock, *J. Immunol.*, **155**:1079-1090 (1995); Frank *et al.*, *Proc. Natl. Acad. Sci. USA*, **92**:7779-7783 (1995)].

Th Jak kinases are characterized by seven conserved domains: two PTK-related domains and five domains with unknown functions [Zi miecki *et al.*, *supra*]. The main difference between Jaks and other protein tyrosine kinases (PTK) is that along with a kinase domain, shown to be active [Wilks *et al.*, *supra*], they also contain a PTK-like domain with substitutions of several residues essential for kinase activity. Thus, the second domain is expected to be inactive as a PTK and probably has some other function. Another feature of this family is the lack of any detectable SH2 or SH3 domains. The functions of the other five regions of homology are also unknown.

Stats represent proteins containing SH2, SH3 and DNA-binding domains [for reviews, see Darnell *et al.*, *supra*; Fu, *Journal of Leukocyte Biology*, **57**:529-535 (1995)]. The highly selective and specific interaction between Stat SH2 domains and the phosphotyrosine containing Stat recruitment sites on the intracellular domains of the cytokine receptors determines which Stats are to be recruited to a particular receptor complex [Heim *et al.*, *Science*, **267**:1347-1349 (1995); Stahl *et al.*, *Science*, **267**:1349-1353 (1995)].

The intracellular domain of each cytokine receptor specifically associates with one or more distinct Jaks. While some Jaks and Stats participate in several cytokine signalling pathways, others are more restricted. In the case of Jaks, Jak2 and, especially Jak1, associate with receptors participating in different apparently unrelated cytokine-receptor systems [for reviews, see Ziemiecki *et al.*, *supra*; Ihle *et al.*, *supra*; Taniguchi, *supra*; Ihle and Kerr, *supra*]. Jak3 appears to be restricted to the ligand receptor systems through its association with the IL-2Rγ_C chain [Johnston *et al.*, *Nature*, **370**:151-153 (1994); Witthuhn *et al.*, *supra*; Russell *et al.*, *Science*, **266**:1042-1045 (1994); Miyazaki *et al.*, *Science*, **266**:1045-1047 (1994); Tanaka *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**:7271-7275 (1994)]. Tyk2 was shown to be activated during IFN-α signalling [Velazquez *et al.*, *supra*; Barbieri *et al.*, *Eur. J. Biochem.*, **223**:427-435 (1994)] and also during CNTF-related cytokine signalling, alb it only in certain cell types [Stahl *et al.*, *Science*, **263**:92-95 (1994); Lütticken *et al.*, *Science*, **262**:89-92 (1994)]. Rec ntly, the activati n of Tyk2 by IL-10 and IL-12 was shown [Finbloom and Winestock, *supra*; Bacon *et al.*, *J. Exp. Med.*, **181**:399-404

(1995); Ho *et al., Mol. Cell. Biol.*, **15**:5043-5053 (1995)]. Thus, th Jaks may contribute to th specificity of signal transduction at some level.

Interferon-γ Receptor Complex

The interferon gamma (IFN-γ) receptor complex consists of at least two receptor components, a ligand binding chain and a signal transducing chain, each of which is a member of the class II cytokine receptor family [Bazan, *Proc. Natl. Acad. Sci. USA*, **87**:6934-6938 (1990); Thoreau *et al., FEBS Lett.* **282**:26-31 (1991)]. Isolation of the two chains of the interferon gamma receptor (IFN-γR) has permitted an analysis of the contributions of each to the signal transduction mechanism. The first chain of the receptor (IFN-γR1) binds ligand [Rashidbaigi *et al., Proc. Natl. Acad. Sci. USA* **83**:384-388 (1986); Aguet *et al., Cell,* **55**:273-280 (1988); Kumar *et al., J. Biol. Chem.* **264**:17939-17946 (1989); Hemmi *et al., Proc. Natl. Acad. Sci. U.S.A.* **86**:9901-9905 (1989); Gray *et al., Proc. Natl. Acad. Sci. USA*, **86**:8497-8501 (1989); Munro and Maniatis, *Proc. Natl. Acad. Sci. USA* **86**:9248-9252 (1989); Cofano *et al., J. Biol. Chem.*, **265**:4064-4071 (1990)]. The second chain of the receptor (IFN-γR2) does not bind ligand by itself but is required for signal transduction [Rashidbaigi *et al.*, 1986, *supra*; Jung *et al., Proc. Natl. Acad. Sci. USA* **84**:4151-4155 (1987), *Somat. Cell Mol. Genet.* **14**:583-592 (1988), *J. Biol. Chem.* **265**, 1827-1830 (1990); Soh *et al., Proc. Natl. Acad. Sci. USA* **90**:8737-8741 (1993), *Cell,* **76**:793-802 (1994); Hemmi *et al., Cell* **76**:803-810 (1994); Kotenko *et al., J. Biol. Chem.* **270**:20915-20921 (1995)]. It was suggested that the two chains of the IFN-γ receptor bind the ligand with a higher apparent affinity than the IFN-γR1 chain alone [Lai, The Mapping of the Murine and the Human Interferon Gamma Receptor. Ph.D. thesis submitted to the Graduate School - New Brunswick, Rutgers, The State University of New Jersey, and the Graduate School of Biomedical Sciences, University of Medicine and Dentistry of New Jersey (1994); Marsters *et al., Proc. Natl. Acad. Sci. USA* **92**:5401-5405 (1995)]. Though the cytoplasmic domains of both IFN-γ receptor subunits lack tyrosin kinase activity, ligand binding is coupled to the inducti n of pr tein phosphorylation, which is essential for receptor function. Jak1 and Jak2 are required for signal transduction by IFN-γ. Further analyses of the interactions have shown that the IFN-γR1 chain binds Jak1 [Igarashi *et al., J. Biol. Chem.*

269:14333-14336 (1994); Sakatsume *et al.*, *J. Biol. Chem.* **270**:17528-17534 (1995); Kotenko *et al.*, 1995, *supra*)] and the intracellular domain of the IFN-γR2 chain brings Jak2 into the signal transduction complex [Kotenko *et al.*, 1995, *supra*]. The only apparent function of the intracellular domain of the IFN-γR2 chain is to bring Jak2 into the signal transduction complex. Upon binding of the ligand, IFN-γ, to the IFN-γR1 chain, activation of Jak1 and/or Jak2 by reciprocal transphosphorylation causes the phosphorylation of IFN-γR1 [Greenlund *et al.*, *J. Biol. Chem.* **268**:18103-18110 (1994); Kotenko *et al.*, 1995, *supra*]. Stat1α, a latent cytoplasmic transcription factor [Schindler *et al.*, *Proc. Natl. Acad. Sci. USA* **89**:7836-7839 (1992)] binds to the phosphorylated IFN-γR1, undergoes tyrosine phosphorylation [(Shuai *et al.*, *Science* **261**:1744-1746 (1993)] and forms homodimers that translocate to the nucleus and initiate transcription of IFN-γ inducible genes (for review see Darnell *et al.*, *Science* **264**:1415-1421 (1994)).

The detailed cell surface events that initiate signaling by IFN-γ have not been clearly elucidated. As with other cytokine receptors, oligomerization upon ligand binding is the first step in the signaling cascade of IFN-γ. IFN-γ is a non-covalent symmetrical homodimer [Ealick *et al.*, *Science* **252**:698-700 (1991)] that binds to IFN-γR1 with a stoichiometry of 1:2 [Fountoulakis *et al.*, *Eur. J. Biochem.* **208**:781-787 (1992); Greenlund *et al.*, *J. Biol. Chem.*, **268**:18103-18110, (1993)]. It is known that a species-specific interaction between the extracellular domains of the IFN-γR1 and IFN-γR2 subunits is essential for signaling [Gibbs *et al.*, *Mol. Cell. Biol.* **11**:5860-5866 (1991); Hibino *et al.*, *J. Biol. Chem.* **267**:3741-3749 (1992); Hemmi *et al.*, *Proc. Natl. Acad. Sci. USA* **89**:2737-2741 (1992)]. The IFN-γR2 subunit does not by itself bind the ligand, but can be crosslinked to IFN-γ when both IFN-γR1 and IFN-γR2 chains are present [Kotenko *et al.*, 1995, *supra*]. Several lines of evidence [Marsters *et al.*, 1995, *supra*; Kotenko *et al.*, 1995, *supra*] suggest that the IFN-γ signaling complex contains two IFN-γR1 chains, and probably two IFN-γR2 chains. The requirement for formation of a heteromeric ternary complex containing two IFN-γR1 chains, two IFN-γR2 chains and one IFNγ homodimer remains unexplained in light of the fact that the IFN-γR1 chain possesses both a Jak1 binding site and a Stat1α recruitment site. Therefore, a heterodimer of IFN-γR1 and IFN-γR2 or a

homodimer of IFN-γR1 alone, upon ligand binding, would be expected to bring into apposition a Jak1 and a Jak2 kinase in the former case, and two Jak1 kinases in the latter case, to activate each other by transphosphorylation. It is not known whether the absolute requirement for two Jaks is unique to the IFN receptor systems, or if it extends to other receptor systems in which two Jaks are activated. Although IL-6 activates Jak1, Jak2, and Tyk2 [Ihle *et al.*, *Annu. Rev. Immunol.*, **13**:369-398 (1995)], only Jak1 is involved in IL-6-induced gp130 tyrosine phosphorylation [Guschin *et al.*, *EMBO J.*, **14**:1421-1429 (1995)].

Erythropoietin Receptor

The erythropoietin (Epo) receptor, EpoR, is a member of the class 1 cytokine receptor subfamily. A single chain appears to encode both ligand-binding and signal-transducing functions. Epo induces homodimerization of the receptor and appears to signal via the dimerized receptor complex [reviewed by Wells, *Curr. Opinion Cell Biol.* **6**:163-173 (1994)]. Jak2 is associated with the cytoplasmic domain of the EpoR and is activated upon ligand-induced dimerization of the receptor [Witthuhn *et al.*, *Cell* **74**:227-236 (1993)]. Strikingly an Arg-to-Cys mutation in the extracellular domain of EpoR results in ligand independent dimerization/oligomerization and constitutive, ligand-independent activation of Jak2 and mitogenesis [Watowich *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**:2140-2144 (1992); Yoshimura *et al.*, *Nature*, **348**:647-649 (1990)].

Interleukin-10 Receptor

IL-10 is a pleiotropic cytokine that plays an important role in the regulation of immune response by controlling the functions of myeloid and lymphoid cells [reviewed in Moore *et al.*, *Annu. Rev. Immunol*, **11**:165-90 (1993)]. Distinct activities of IL-10 on T_H1- and T_H2-related immune functions suggest a possible role in controlling development of the class of an immune response. IL-10's multiple activities have been shown so far to include: the ability to inhibit macrophage activation and suppress cytokine synthesis by activated T cells and NK cells by blocking the ability of macrophages to act as antigen-presenting or costimulatory cells; and costimulation of proliferation and differentiation of B cells, mast cells, and thymocytes. IL-10 can inhibit dendritic and antigen

presenting cell (APC) functions. It can also inhibit cytokine synthesis and eff ctor functions of T_H1 cells. IL-10 has distinct pathways for its activiti s, for example on NK cells. Thus, although both IL-4 and IL-10 inhibit IL-2 induced synthesis of IFN-γ and TNF-$\alpha$by PBMC, IL-10 does not affect IL-2-induced NK or PBMC proliferation, nor does it affect IL-2-induced LAK activity. The effects of IL-10 on IFN-γ suppression are mediated by CD14 + cells (monocytes/macrophages) [Hsu *et al.*, *Int. Immunol.* **4**:563-569 (1991)].

The IL-10 receptor binding chain was isolated [Liu *et al.*, *J. Immunol.* **152**:1821-1829 (1994)] and binds to IL-10, but surprisingly does not seem to bind vIL-10 [*See* Ho and Moore, *Therapeutic Immunology*, 1:173-185 (1994)]. Recently, as described herein and more fully in co-owned, co-pending application Serial No. 08/683,743, filed on the same date as this application, having attorney docket no. 601-1-050, entitled "CYTOKINE RECEPTOR SIGNAL TRANSDUCTION CHAIN" by Sidney Pestka and Serguei Kotenko, which is incorporated herein by reference in its entirety, the protein CRFB4 [Lutfall *et al.*, *Genomics*, **16**:366-373 (1993)] was identified as the second chain of the functional IL-10 receptor, which is necessary for signal transduction.

Non-MHC-Restricted T Cells

Previous reports describe chimeric receptors comprising extracellular domains capable of binding to an antigen in a non-MHC restricted manner, for example an immunoglobulin antigen binding domain, a transmembrane domain, and a cytoplasmic domain of a T cell receptor complex or Fc_ϵ receptor-1 gamma chain capable of activating a signalling pathway have been described (Capon *et al.*, U.S. Patent No. 5,359,046, issued October 25, 1994). Functional antigen-specific receptors were generated in B cells [Sanchez *et al.*, *J. Exp. Med.* **178**:1049-1055 (1993)] and T cells [Burkhardt *et al.*, *Mol. Cell. Biol.*, **14**:1095-1103 (1994)] by creating a chimeric receptor by fusing the Igα and Igβ signal transduction chains to IgM. These reports demonstrated that the molecular structures involved in antig n rec ptor signaling pathways are conserved between T and B cells.

One problem with the chimeric receptor constructs of the prior art is that

although they permit creation of non-MHC restrict d T cells, the signal transduction machinery may not extend to other effector cells that do not respond to the same molecule structures involved in receptor signalling that are employed by B cells and T cells. Thus, there is a need in the art for mediating signal transduction through an entirely different set of pathways.

There is a further need in the art to identify alternative signalling routes in subjects who are deficient in one pathway or another. For example, there is a need to compensate for the lack of Jak3 in some individuals, who consequently present with a SCID phenotype.

These and other deficiencies of the prior art are addressed by the present invention, which particularly relates to activation via cytokine and growth factor receptor signal transduction pathways through binding of alternative ligands to chimeric receptors.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

The present invention advantageously, and unexpectedly, harnesses cytokine and growth factor receptor cellular activation pathways for responses to different receptor ligands, thus enabling reconstitution of cellular activation to a ligand, such as a cytokine or growth factor, where some deficiency prevents activation by the native pathway; targeting of effector cells to tumors, virally infected cells, or autoimmune cells; and creating new cytokine or growth factor sensitivities in responder cells, *e.g.*, to develop an assay system in which cells not normally responsive to a given cytokine are rendered responsive.

Accordingly, the invention provides a chimeric receptor for cellular activation comprising an extracellular domain capable of binding to a cognat molecule, and a cytokine intracellular domain capable of interacting with a Jak-Stat prot in, thereby participating in signal transduction, with the proviso that the

cytokine intracellular domain is not naturally associated with the extracellular domain. Preferably, the cytokin intracellular domain interacts with a Jak protein selected from the group consisting of Jak1, Jak2, Jak3, and Tyk2. In another embodiment, preferably the cytokine intracellular domain interacts with a Stat protein selected from the group consisting of Stat1α, Stat1β, Stat2, Stat3, Stat4, Stat5, and Stat6.

The invention provides for a wide variety of extracellular domains for the chimeric receptors, provided that the binding activity of the extracellular domain accomplishes receptor dimerization or oligomerization. Thus, in one embodiment, the chimeric receptor extracellular domain is selected from the group consisting of a cytokine and a growth factor. A cell that expresses a chimeric receptor of this aspect of the invention would be targeted to cells bearing the cognate cytokine or growth factor receptor. In specific embodiments of this aspect of the invention, the cytokine is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-10, and interleukin-12.

Alternatively, the chimeric receptor may employ an extracellular domain that is a receptor ligand binding extracellular domain. For example, the receptor ligand binding extracellular domain is selected from the group consisting of a cytokine receptor ligand binding domain, and a growth factor receptor ligand binding domain. In this embodiment of the invention, where a deficiency in the native signal transduction pathway prevents activation to a cytokine or growth factor, substitution of a different cytoplasmic signal transduction domain for the ineffective domain in a chimeric receptor construct can circumvent the deficiency. Alternatively, cells that normally do not respond to a given cytokine or growth factor can be made responsive by substituting cytoplasmic signal transduction domains from a growth factor or cytokine receptor to which the cells responds for the native cytoplasmic domain on the naturally non-responsive receptor. The Examples, *infra*, demonstrate these asp cts of the invention. In specific embodim nts, the cyt kine rec ptor ligand binding domain is an extracellular domain selected from the group consisting of Interferon-α, Interferon-β, Interferon-γ, interleukin-1, interleukin-2, interleukin-3, interleukin-4,

interleukin-6, interleukin-8, interleukin-10, interleukin-12, and rythropoi tin.

In yet another embodiment, the chimeric receptor employs an immunoglobulin antigen binding domain, *e.g.*, a heavy chain, a light chain, a heavy chain-light chain dimer, a single chain F_v, as the receptor ligand binding extracellular domain.

Naturally, the present invention provides a genetically engineered nucleic acid encoding a chimeric receptor for cellular activation comprising an extracellular domain capable of binding to a cognate molecule, and a cytokine intracellular domain capable of interacting with a Jak-Stat protein, thereby participating in signal transduction, with the proviso that the cytokine intracellular domain is not naturally associated with the extracellular domain. Thus, the invention includes a nucleic acid encoding each of the chimeric receptors set forth above.

Preferably, the nucleic acid of the invention is capable of expressing the chimeric receptor protein. Accordingly, the invention provides an expression vector comprising the nucleic acid operably associated with an expression control sequence. Preferably, the expression control sequence, *e.g.*, promoter, provides for high level expression in the cell in which the expression vector is introduced.

In addition to the expression vectors of the invention, also provided is a genetically engineered host cell comprising the expression vector. Suitable host cells include bacterial, yeast, and eukaryotic cells. Preferably the host cell is a mammalian cell. In one aspect, the host cell is an effector cell, and the expression vector is introduced into the effector cell *ex vivo* or *in vivo*, as set forth below. In another embodiment, the cell is a responder cell, and the expression vector is introduced into the effector cell *ex vivo* or *in vivo*, as set forth below. In yet another embodiment, the host cell is a cultured cell, which provides a novel cytokine or growth factor assay system.

Using the chim ric receptors ncoded by the expression vectors, th invention provides a method for reconstituting responsiveness to a soluble factor comprising administering the expression vector to c lls of a host animal lacking

responsiveness to a soluble factor, wherein the chimeric receptor encoded by the expression vector comprises an extracellular domain that binds to the soluble factor. Administration of the expression vector can be accomplished *ex vivo* or *in vivo*.

In still another aspect, the invention provides targeted effector cells, which are prepared by introducing the expression vector into effector cells, wherein the chimeric receptor encoded by the expression vector comprises an extracellular domain which is an antibody antigen binding domain. In specific embodiments, the effector cells are selected from the group consisting of natural killer cells, lymphokine activated killer cells, cytotoxic T cells, macrophages/monocytes, neutrophils, basophils, and polymorphonuclear leukocytes. The effector cells of the invention are useful in a method for treating a disease or disorder characterized by disregulation of a cellular activity; the invention contemplates administering the effector cells targeted to the cell which is undergoing disregulation. The disease or disorder may be, but is by no means limited to, viral infection, cancer, inflammatory disease, and autoimmune disease.

In another embodiment, the invention provides targeted effector cells comprising an expression vector that encodes a chimeric receptor having an extracellular domain which is selected from the group consisting of a cytokine and a growth factor. More particularly, the effector cells may be selected from the group consisting of natural killer cells, lymphokine activated killer cells, cytotoxic T cells, macrophages/monocytes, neutrophils, basophils, and polymorphonuclear leukocytes. Other effector cells may be chosen. By analogy with the cells that are modified to express an immunoglobulin chimeric receptor, the invention provides a method for treating a disease or disorder characterized by disregulation of a cellular activity comprising administering the effector cells containing a growth factor or cytokine chimeric receptor targeted to the cell which is undergoing disregulation, which cell undergoing disregulation expresses the receptor for the growth factor or the cytokine. As described above, the disease or disorder may be, but is not limited to, viral infection, cancer, inflammatory disease, and autoimmune disease.

DESCRIPTION OF THE DRAWINGS

FIGURE 1. *Structure of Chimeric Receptors.* Hu-IFN-γR1 (γR1) and Hu-IFN-γR2 (γR2) are the intact chains of the human IFN-γ receptor complex. All chimeric receptors have the extracellular domain of the human IFN-γR2 and the transmembrane and intracellular domains of different human receptors: γR2/γR1, Hu-IFN-γR1; γR2/αR1, Hu-IFN-αR1 [Uzé, *et al.*, *Cell* **60**:225-234 (1990)]; γR2/αR2, Hu-IFN-αR2 [Novick *et al.*, *Cell* **77**:381-400 (1995)]; γR2/CRF, CRFB4 [Lutfalla *et al.*, *Genomics* **16**:366-373 (1993)]; γR2/γ_C, IL-2 receptor γ_C chain [Takeshita *et al.*, *Science* **257**:379-382 (1992)]. Although it was reported that the short form of Hu-IFN-αR2 we used binds Jak2 [Novick *et al.*, *supra*], a "?" is placed under the γR1/αR2 chimera because only the long form of Hu-IFN-γR2 is functional [Lutfalla *et al.*, *EMBO J.* **14**:5100-8 (1995); Domanski *et al.*, *J. Biol. Chem.* **270**:21606-11 (1995)].

FIGURE 2. *Expression of Chimeric Receptors in COS-1 Cells.* COS-1 cells were transfected with the expression vectors encoding different chimeric receptors: γR2, γR2/αR1, γR2/αR2, γR2/CRF, γR2/Rγ_C, γR2/γR1 and γR2/γR1t_{456}. The intracellular domain of the chimera γR2/γR1t_{456} represents the intracellular domain of Hu-IFN-γR1 terminated by premature stop codon after amino acid 456 [Kotenko *et al.*, *J. Biol. Chem.* **270**:20915-21 (1995)]. Cells were harvested after three days and lysed as described under "Experimental Procedures" in Example 1. Lysates were resolved on SDS-PAGE, transferred to PVDF membranes and Western blots probed with anti-Hu-IFN-γR2 antibodies.

FIGURE 3. *Covalent Cross-linking of [^{32}P]IFN-γ to the Receptors.* Cells were harvested and incubated with [^{32}P]Hu-IFN-γ with or without addition of a 200-fold excess of unlabeled Hu-IFN-γ and cross-linked as described under "Experimental Procedures" in Example 1. The extracted ligand:receptor complexes were analyzed on a 7.5% SDS-polyacrylamide gel. The cell lines indicated on the figure repres nt the 16-9 hamster cells (expr ssing the xogenous Hu-IFN-γR1 chain) transfected with plasmids encoding differ nt s cond recept r chains: γR2; γR2/γR1, γR2/γR1t_{456}, γR2/αR1, γR2/αR2, γR2/CRF and γR2/γ_C. The arrows designate the complexes of Hu-IFN-γ with diff r nt

receptors: γR1, γR2, γR2/αR1, γR2/αR2, γR2/CRF, γR2/γ_C, γR2/γR1 and γR2/γR1t_{456}.

FIGURE 4. *Induction of HLA-B7 Surface Antigen.* Induction of HLA-B7 surface antigen in cells treated with Hu-IFN-γ. The parental 16-9 cells express only exogenous Hu-IFN-γR1 (A); the other cells express both γR1 and various receptor chains: γR2 (B); γR2/γR1 (C); γR1/γR1t_{456} (D); γR2/αR1 (E); γR2/αR2 (F); γR2/CRF (G); and γR2/γ_C (H). The γR2/γ_C + Tyk2 cell line represents 16-9 cells cotransfected with expression vectors encoding γR2/γ_C and human Tyk2. HLA-B7 antigen was detected by treatment of cells with mouse anti-HLA monoclonal antibody W6/32 followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The cells were then analyzed by cytofluorography. The unstippled areas represent cells not treated with IFN; stippled areas represent cells treated with 1000 units/ml of Hu-IFN-γ. Relative fluorescence values are shown on a log scale as described [Hibino *et al., J. Biol. Chem.* **267**:3741-3749 (1992)].

FIGURE 5. *Tyrosine Phosphorylation of Jaks upon IFN-γ Treatment.* Untreated and Hu-IFN-γ treated cells were lysed and immunoprecipitated with anti-Jak2 (A), anti-Tyk2 (B, D and E), anti-Jak3 (F) and anti-phosphotyrosine antibodies (D) as described under "Experimental Procedures" in Example 1. The cell lines are as indicated on the figure and defined in the legends to Figure 3 and 4. In addition, the γR2/X + Tyk2 cell lines represents 16-9 cells cotransfected with the designated expression vectors encoding γR2/X receptors and human Tyk2; and the γR2/X + Jak3 cell line, 16-9 cells cotransfected with designated expression vectors encoding γR2/X receptors and Jak3. Immunoprecipitates were resolved on SDS-PAGE, transferred to PVDF membranes and probed with various antibodies: anti-phosphotyrosine antibodies, first panels in A, B, D, E and F; with anti-Jak2 antibodies, second panel in A; with anti-Tyk2 antibodies, C, D and second panels in B and E; and with anti-Jak3 antibodies, second panel in F.

FIGURE 6. *Electrophoretic Mobility Shift Assay (EMSA).* EMSAs were performed as described under "Experimental Procedures" in Example 1 with the 22 base pair labelled sequence containing the Stat1α binding site corresponding

to the GAS element in th promoter region of the human IRF-1 gene with nuclear extracts from the cells indicated on the figure and d fined in the legend to Figure 4. In addition, HEp-2 cells, a human epidermoid larynx carcinoma cell line, were used as a positive control. Supershift assays were performed with specific anti-Stat1α antibodies. The position of the Stat1α DNA-binding complexes are indicated by the arrow.

FIGURE 7. *Model of the Signal Transduction by IFN-γ.* After oligomerization of the IFN-γ receptor chains caused by ligand binding, the IFN-γ homodimer binds to two IFN-γR1 chains which in turn brings two associated IFN-γR2 (AF-1) chains and all the associated components (Jaks and Stats) into the complex [Kotenko *et al., supra*]. The interaction of Jaks with the intracellular chains initiates the cascade of events resulting in activation of specific Stats as described in the text. JAS, represents Jak association site; SRS, Stat recruitment site; ST, signal transducing receptor; HR, helper receptor; PTK, protein tyrosine kinase; γR2/X, chimeric receptor with the extracellular domain of the IFN-γR2 and the intracellular domains of various receptors swapped for the intracellular domain of the Hu-IFN-γR2 chain.

FIGURE 8. *Map of expression vector pEF2.*

FIGURE 9. *Induction of HLA-B7 Surface Antigen in Hamster Cells by IL-10.* (A) The hamster cells stably transfected with the Hu-IL-10R/γR1 chimeric receptor chain were treated with IL-10 or left untreated. (B) The hamster cells stably transfected with both IL-10R/γR1 chimeric receptor and CRFB4 chains were treated with IL-10 or left untreated. The cells were then analyzed by flow cytometry as described. The unstippled areas represent cells left untreated with IL-10; stippled areas represent cells treated with IL-10. HLA-B7 antigen was detected by treatment of cells with mouse anti-HLA monoclonal antibody W6/32 followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Relative fluorescence values are shown on a log scal as described [Hibino *et al., J. Biol. Chem.*, **267**:3741-3749 (1992)].

FIGURE 10. *Schematic representation of native and chimeric receptors.* EpoR,

γR1 and γR2 represent the wild-type human rythropoietin receptor, Hu-IFN-γR1 and Hu-IFN-γR2, r spectively. The various chimeric constructs were mad by joining the extracellular domain (top) with the transmembrane and cytoplasmic domains (bottom) of the receptors indicated. The EpoR(p91) and EpoR/γR2(p91) chimeras contain the five-amino acid Stat1α recruitment site of Hu-IFN-γR1 at the 3′ end of the intracellular domain of EpoR and IFN-γR2 respectively.

FIGURE 11. *Induction of class I MHC surface antigens.* Induction of class I MHC surface antigens by Hu-IFN-γ or Epo, as indicated, of the parental 16-9 cells which expresses only Hu-IFN-γR1 (panel A), and of 16-9 cells expressing Hu-IFN-γR1 along with various transfected receptor chains: IFN-γR2 (panel B), γR2/EpoR (panel C), EpoR (panel D), EpoR/γR1 (panel E), both EpoR/γR1 and EpoR/γR2 (panel F), γR1/EpoR(p91) (panel G) and EpoR(p91) (panel H). Cells were treated with Hu-IFN-γ at 1000 units/ml, or Epo at 10 units/ml for 72 hours as described. Class I MHC antigens were detected by treatment of cells with mouse anti-human-HLA-B7 monoclonal antibody (W6/32) followed by treatment with FITC-conjugated goat anti-mouse IgG and cytofluorographic analysis. The unshaded regions represent untreated cells and shaded regions represent cells treated with Hu-IFN-γ (panels A-C, G) or Epo (panels D-F, H).

FIGURE 12. *Induction of expression of class I MHC surface antigens as a function of interferon concentration.* Cells were treated with Hu-IFN-γ at concentrations of 0, 1, 10 and 100 units/ml. Class I MHC antigens were detected as described in the legend to Figure 11. Relative fluorescence values are based on the mean fluorescence of cell populations (n = 10,000).

FIGURE 13. *Induction of class I MHC antigens as a function of Epo concentration.* The induction of expression of class I MHC antigens as a function of Epo concentration was assessed by treating cells with varying concentrations of Epo at 0, 0.1, 1, 10 and 50 units/ml. Relative fluorescence values are based on the mean fluorescenc of cell populations (n = 10,000).

FIGURE 14. *Electrophoretic mobility shift assays of cells expressing chimeric receptors.* Transfected clones of 16-9 cells stably xpressing native IFN-γR2 or

chimeric IFN-γR2/EpoR receptor subunits w re induced with 1,000 units/ml of IFN-γ. Whole cell extracts were prepared, incubated with 32P-labeled GAS probe, complexes resolved by separation on 5% polyacrylamide gels and detected by autoradiography. Competition experiments contained a 100-fold molar excess of unlabeled GAS oligonucleotide. Supershift assays were performed by the addition of 0.1 μg of antibody specific to Stat1α (anti-Stat1α) or Stat5 (anti-Stat5). Arrow A marks the position of the Stat1α complex, B marks the position of the Stat5 complex activated only in those cells expressing the intracellular domain of EpoR. Arrows C and D designate the positions of the Stat1α and Stat 5 complexes supershifted by their respective antibodies.

FIGURE 15. *Electrophoretic mobility shift assays of cells expressing chimeric receptors.* The 16-9 cells were transfected with EpoR/γR1 chimeric receptor or co-transfected with EpoR/γR1 and EpoR/γR2 chimeric constructs. A clonal population of transfected cells were induced with erythropoietin at 50 units/ml for 15 minutes at 37°C. Whole cell extracts were made and the electrophoretic mobility shift assay performed. As shown in the figure, induction with Epo causes activation of Stat1α in cells expressing EpoR/γR1 alone as well as in those cells expressing both EpoR/γR1 and EpoR/γR2. Addition of anti-Stat1α antibody to the reaction mixture causes the Stat1α complex to be shifted.

FIGURE 16. *Phosphorylation of Jak1 and Jak2 kinases.* Untreated and Hu-IFN-γ or Epo treated cells were lysed and immunoprecipitated with monoclonal anti-phosphotyrosine antibodies (panels A and C) or polyclonal anti-Jak1 (panel B) or anti-Jak2 antibodies (panel D) as described in "Materials and Methods" in Example 3. The cell lines are indicated on the figure and are defined in the legend to Figure 10. Immunoprecipitates were resolved on SDS-PAGE, transferred to PVDF membrane and probed with anti-Jak1 (panels A and B) or anti-Jak2 (panels C and D) antibodies.

FIGURE 17. *Schematic representation of receptor complexes.* (A) represents the IFN-γR1 homodimer bound to IFN-γ. The cytoplasmic domains of the two chains ar too far apart to permit transactivation of the two Jak1 kinases. (B) represents the active heter meric IFN-γ receptor complex with two IFN-γR1 and

two IFN-γR2 subunits per complex. The associated Jak2 and Jak1 kinases activate one another by transphosphorylation, with subsequent phosphorylation and dimerization of Stat 1α. (C) depicts the EpoR/γR1 homodimer which, unlike the IFN-γR1 homodimer, permits transactivation of the two Jak1 kinases. (D) illustrates the structure of the heterodimer of EpoR/γR1 and EpoR/γR2 which is the putative active receptor complex.

FIGURE 18. *Induction of HLA-B7 Surface Antigen.* The parental 16-9 cells (A) and the H1/γR1 + L1/γR2 cells (B), 16-9 cells co-transfected with two expression plasmids encoding chimeric antibody-receptor molecules L1/γR2 (V_L domain of light chain of cc49 antibody fused to the transmembrane and intracellular domains of human IFN-γR2 chain) and H1/γR1 (V_L domain of heavy chain of cc49 antibody fused to the transmembrane and intracellular domains of human IFN-γR1 chain) were treated (stippled area) or untreated (unstippled areas) with paraformaldehyde fixed MCF-7 clone 4C10 cells for 72 hours. HLA-B7 antigen was detected by treatment of cells with mouse anti-HLA monoclonal antibody W6/32 followed by treatment with FITC-conjugated goat anti-mouse IgG. The cells were then analyzed by cytofluorography. Relative fluorescence values are shown on a log scale.

FIGURE 19. *Expression of Chimeric Antibody-Receptor molecules.* Two expression plasmids encoding chimeric antibody-receptor molecules L1/γR2 and H1/γR1 were co-transfected into mouse CTL1-2 cells (B). The expression of the chimeric antibodies on the cell surface was detected by treatment of cells with FITC-conjugated goat anti-mouse IgG. The H1/γR1 + L1/γR2 16-9 cells (A) described in Figure 18 were used as a positive control, as they were shown to be antigen-responsive and thus express functional chimeric antibody-receptor molecules on the cell surface. Unstippled areas represent untransfected cells and stippled areas represent transfected cells. The cells were then analyzed by cytofluorography. Relative fluorescence values are shown on a log scale.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention harnesses cytokin and growth factor

r ceptor cellular activation pathways for responses to different receptor ligands. The chimeric receptors enable reconstitution of cellular activation to a ligand, such as a cytokine or growth factor, where some deficiency prevents activation by the native pathway. They allow for targeting of effector cells to tumors, virally infected cells, or autoimmune cells. The chimer receptors further provide for creating new cytokine or growth factor sensitivities in responder cells, *e.g.*, to develop an assay system in which cells not normally responsive to a given cytokine are rendered responsive, and for testing the activation pathways of unknown cytoplasmic domains.

Cell surface receptors exist for small and large ligands. They are naturally occurring on eukaryotic and prokaryotic cells. According to the invention, chimeric cell surface receptors are linked to signal transduction mechanisms, thus creating functional synthetic receptors with customized ligand recognition and cellular activation pathways. The chimeric receptors can be designed to interact with any ligand (*i.e.*, almost any chemical moiety) and generate virtually any growth factor or cytokine receptor signals. For example, as shown in specific examples, *infra*, cells reactive to IFN-γ can be engineered to respond via the IFN-γ activation pathway in response to exposure to a cytokine (in specific examples, erythropoietin and IL-10) or an antigen (in a specific example, the tumor-associated antigen TAG72).

Expression of a chimeric receptor or chimeric receptor complex in cytotoxic cells, for example, receptors generated to interact specifically with tumor associated antigens, allows creation of cytotoxic cells that can then be used to eliminate cancer cells. Receptors generated specifically to interact with viral cell surface antigens can be used to eliminate these cells and effectively cure chronic viral infections such as AIDS, hepatitis, herpes, papillomatosis, and many other infectious diseases. Furthermore, by eliminating specific cytotoxic cells recognizing self-antigens, autoimmune disease such as multiple sclerosis, rheumatoid arthritis, and other autoimmune disease could be attacked by this t chnology. The ability to harness the signal transduction machine of Class I and II cytokin r ceptors, which involves highly complex oligomerization and activation of Jak/Stat prot ins, with chimeric receptor ctodomains, was wholly

unpredictable and unexpected.

Antibodies can be generated to virtually any antigen or hapten. Accordingly, the heavy and light chains of an antibody molecule represents a specific antigen-antibody reason comparable to the ligand-receptor interaction in its specificity. The range of specificities and chemical constants (*e.g.*, association constants, forward and reverse kinetic constants) for both these reactions are also comparable and represent a wide range of possibilities. For this reason, it seemed feasible that the use of specific antibodies could be used to generate synthetic receptors that could be coupled to the cytokine or growth factor signal transduction pathways of cells. By combining the appropriate heavy and light chains with specific cytoplasmic receptor chains, a wide variety of signal transduction pathways can be activated by the combinatorial choice of the cytosolic domains. In this manner, it would be possible to generate a synthetic receptor to any antigen or hapten and to couple this to virtually any cytokine or grow factor signal transduction array to activate specific biological pathways within the cell. By modifying these "ectodomains" and internal domains synthetically, one can modulate and develop an incomparable greater variety of synthetic receptors.

Two major specific recognition components of the immune system are antibodies produced by B-lymphocytes and the T-cell receptor of T-lymphocytes. The outline noted above for antibodies can also be applied to the T-cell receptor. By using specific chimeric T-cell receptors (T-cell receptor ectodomain coupled to different intracellular domains) that can recognize specific antigens in combination with the appropriate MHC molecules, it would be possible to upregulate and down regulate immune response, or to develop a parallel cellular response.

It is also possible to use ligands where appropriate as extracellular domains. For example, in T-cell leukemia th IL-2 receptor is overexpressed on these c lls. Antibodi s to the IL-2R (with radioisotopes, toxins, or cytotoxic molecules attached) are being used to kill th se cells to treat T-cell leukemia. To apply synthetic receptors of th invention to kill these cells, IL-2 itself would be

coupled (*i.e.*, fuse th IL-2 ligand as an ctodomain) to th appropriate receptor intracellular domains (*e.g.*, IFN-γR complex) to introduce into effector cells, such as cytotoxic T-cells, NK cells, and/or macrophages so that they are activated on interaction with the target T-cell leukemia cell, and thus kill the T-cell leukemia cells.

By modifying the intracellular domains of the receptors by the introduction of different components parts of various receptors, synthetic internal domains to generate customized signals can be constructed. By introducing these receptors into the appropriate cells (*e.g.* cytotoxic cells) cytotoxic or other effector response against any target cells can be generated. Response from one cytokine or growth factor can be converted to those of another to responses of another.

Combining the synthetic receptors and the desired growth factor or cytokine signal transduction mechanism to be activated with appropriate cells allows one to generate new cells with new and desired properties. For example, with the use of antibodies generated against tumor associated antigens (TAA) used as the ectodomain of the receptor and the activation of cellular signals with a cytotoxic cells such as a CD8 T-cell, natural killer (NK) cell, macrophage, or TIL cell, new cellular entities could be generated that will specifically target tumor cells to which the ectodomains of the antibodies are targeted. In a specific embodiment, effective chimeric receptors prepared from an anti TAG72 antigen antibody ectodomain and IFN-γ1R and 2R signal transduction domains.

In another embodiment, multiple signal domains can be used. In this case several pathways known to enhance cytotoxic activity could be activated in a single cell with the same set of heavy and light chains by introducing a combinatorial set of endodomains attached to the heavy and light chains that serve as the ectodomains of these new receptors. Such cells introduced into animals or patients with tumors expressing the tumor antigens will be activate when these c lls interact with the tumor cell. Soluble forms of the TAA present in the serum will not activate the cells because the receptors requir dim rization to initiate th signal transduction pathway. Cells can be developed

to downregulate immune responses by directing (targeting) the cytotoxic cells against specific lymph cyt s generating th autoimmun response. The principles developed can be utilized for a variety of therapeutic studies.

In a further specific example, the CRFB4 transmembrane chain supports IL-10-induced signal transduction with the chimeric IL-10R_{EC}/IFN-γR1$_{IC}$ receptor. This chimeric receptor was used to test the model that IL-10 would produce a IFN-γ-like response if the IFN-γR1 intracellular domain were coupled to the intracellular domain of the IFN-γR1 chain. It was also used to test the hypothesis that the IFN-γR2 chain as a helper receptor is required only to bring a second kinase protein into the complex.

This technology also provides a new method for assessing whether receptor chains are part of a specific complex by forming chimeric receptors and evaluation cellular activation.

Various terms are used in this specification, which are more fully defined as follows:

The term "chimeric receptors" refers to a non-naturally occurring receptor molecule having an extracellular domain capable of binding to a ligand or a receptor; a transmembrane domain that anchors the chimeric receptor in the cell membrane; and a cytoplasmic domain that is involved in signal transduction by activation of the Jak/Stat pathway. In the nomenclature convention adopted for this application, the extracellular domain is identified, followed by a slash, followed by the identifier for the cytoplasmic domain. Thus, EpoR/IFN-γR1 refers to a chimeric receptor having an erythropoietin receptor extracellular domain, and an interferon-γ receptor-1 chain cytoplasmic domain.

The term "extracellular domain" refers to the part of the chimeric receptor that is locat d on the outside of th cell, and which therefor is capable of binding soluble ligand, such as a cytokine or growth factor, or binding to a binding partner on another cell, such as a target cell. Synonyms for th t rm extracellular domain used in this specification include "ectodomain," a subscript

"EC," and "extracyoplasmic domain." Examples of extracellular d mains of the invention include, but are not limited to, membrane immunoglobulin/single chain membrane immunoglobulin; a ligand binding domain of a cytokine or growth factor receptor; and a cytokine or growth factor, or receptor-binding domain thereof.

The term "cytokine" as used herein refers to cytokines, lymphokines, and other soluble protein mediators of intercellular signalling that are involved in cell growth, recruitment, inflammation, immunity, differentiation, and repair. These include, but are not limited to, T-cell-derived cytokines (interleukin (IL)-2, Il-3, Il-4, Il-5, IL-6, IL-9, IL-10, interferon (IFN)-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, and TNF-β) [Howard *et al.*, "T-Cell-Derived Cytokines and Their Receptors," In *Fundamental Immunology, Third Edition*, W. Paul, Ed., Raven Press: New York, pp. 763-800 (1993)]; pro-inflammatory cytokines produced predominantly by macrophages (IL-1, TNF, IL-6, IFN-α, IFN-β, platelet-derived growth factor (PDGF), and chemokines -- IL-8, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and macrophage chemotactic and activating factor (MCAF/MCP-1), RANTES, MGSA/GRO) [Durum and Oppenheim, "Proinflammatory Cytokines and Immunity," In *Fundamental Immunology, Third Edition*, W. Paul, Ed., Raven Press: New York, pp. 801-835 (1993)]; and other cytokines (IL-10, IL-11, IL-12, etc.). As used herein, the term "cytokine" is inclusive of "lymphokine."

The term "growth factor" refers to soluble intercellular signalling proteins that induce cell growth or differentiation, or both. Growth factors include, but are by no means limited to, erythropoietin (Epo), colony stimulating factor (CSF; GM-CSF, G-CSF, M-CSF), nerve growth factor (NGF), epidermal growth factor (EGF), transforming growth factor (TGF)-α, TGF-β, fibroblast growth factor (FGF), growth hormone (GH), platelet-derived growth factor (PDGF), somatomedins, and the like [see generally Darnell *et al.*, *Molecular Cell Biology*, Scientific American Books/W.H. Freeman and Company: New York (1986)].

The t rm "cyt kin or growth factor receptor" refers to th receptor mol cule sp cific for the cytokines and growth factors described herein. R ceptors are

described in the references cited above, and are well known in the art.

The cytokines/growth factors and corresponding receptors described herein may be from any species of animal, preferably a species of mammal, and most preferably are human. Also contemplated are cytokines/growth factors and corresponding receptors from domestic animals, such as feline or canine sources, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine sources, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, etc., avian species, such as chickens, turkeys, songbirds, etc., *i.e.*, for veterinary medical use or in *in vitro* assay systems.

A "binding partner on another cell" is a receptor, cell adhesion molecule, or other integral membrane protein that is specifically recognized by a chosen extracellular domain of a chimeric receptor.

The term "cytoplasmic domain" is used herein to refer to the part of the chimeric receptor that is located in the cytoplasm of the cell, *i.e.*, inside the cell. This domain contains any Jak/Stat recognition sites, and is involved in signal transduction upon binding of the chimeric receptor to its ligand or binding partner on another cell. Synonyms for the term cytoplasmic domain used in this specification include "endodomain," "intracellular domain," "cyotsolic domain," and "signal transduction domain." Cytoplasmic domains of the invention may be obtained from any cytokine receptor or growth factor receptor; preferably a Type I or Type II cytokine receptor. Specific embodiments, detailed herein, include the IFN-γR1 cytoplasmic chain, the IFN-γR2 cytoplasmic chain, EpoR cytoplasmic chain, and the CFRB4 cytoplasmic chain. Clearly, other cytoplasmic chains (*i.e.*, domains) from similar growth factor or cytokine receptors can be used as well, *e.g.*, IFN-αR, IFN-βR, etc.

The term "receptor complex" refers to the chimeric receptor, with or without accessory proteins -- including other chimeric receptors -- non-covalently associated in the cell membrane upon binding to ligand. Upon formation of a receptor complex, which may be a chimeric receptor homodimer or homo-

oligomer, *e.g.*, an EpoR/IFN-γR1 heterodimer (Example 3, *infra*); a heterodimer or hetero-oligomer comprising the chimeric receptor and an endogenous receptor complex protein, *e.g.*, IL-10R/IFN-γR1 and CRFB4 (Example 3, *infra*); a heterodimer or hetero-oligomer comprising two different chimeric receptors, *e.g.*, EpoR/γR1 and EpoR/γR2 (Example 3, *infra*), and an apparent heterotetramer comprising two chains of H1/IFN-γR1 and two chains of L1/IFN-γR2 forms (Example 4, *infra*).

The term "effector cell" as used herein refers to a cell that, upon activation, engages in some action, such as cell-mediated immunity, preferably by migrating to the site of a target cell or target tissue. Preferred effector cells of the invention include, but are by no means limited to, helper T cells [Fitch *et al.*, "T-Cell-Mediated Immune Regulation," In *Fundamental Immunology, Third Edition*, W. Paul, Ed., Raven Press: New York, pp. 733-761 (1993)], cytolytic or cytocidal lymphocytes (including cytotoxic T cells, lymphokine activated killer (LAK) cells, and natural killer (NK) cells) [Berke, "The Functions and Mechanisms of Action of Cytolytic Lymphocytes," In *Fundamental Immunology, Third Edition*, W. Paul, Ed., Raven Press: New York, pp. 965-1014 (1993)], phagocytic and inflammatory cells (including mononuclear phagocytes -- macrophages and monocytes, and polymorphonuclear phagocytes -- neutrophils, eosinophils, and basophils) [Greenberg and Silverstein, "Phagocytosis," In *Fundamental Immunology, Third Edition*, W. Paul, Ed., Raven Press: New York, pp. 941-964 (1993)] mast cells, granulocytes, etc.

The term "target cell" refers to a cell having a binding partner on the cell surface that is capable of recognition. Preferred target cells of the invention include tumor cells, virally infected cells, autoimmune cells, and inflammatory cells.

The term "responder cell" refers to a cell that is activated, *e.g.*, produces a soluble factor, such as a cytokine or lymphokine, proliferates, differentiates, transcribes a gene, engages in some activity in response to activation via a chimeric receptor. Examples of responder cells include, but are not limited to, animal cells, such as CHO, RI.I, B-W, and L-M cells, African Green Monkey kidney cells (*e.g.*, COS 1, COS 7, BSC1, BSC40, and BMT10), human cells

(such as HeLa), and hybrid cells (*e.g.*, 16-9 CHO/human hybrid cells) in tissue culture. Responder cells also include animal cells that express a chimeric receptor of the invention *in vivo*, *e.g.*, T cells or B cells that express a chimeric IL-2 receptor γ_c chain extracellular domain and an intracellular domain that would recruit Tyk2, Jak1, or Jak2, *e.g.*, CRFB4. Thus, any cell that normally responds to a cytokine or growth can be modified to respond to that cytokine or growth factor by an alternative activation pathway.

Antibody Extracellular Domains

According to the invention, a monoclonal antibody to any molecule, such as but not limited to a viral antigen, bacterial antigen, tumor antigen, etc., may be employed to provide a membrane immunoglobulin/single chain immunoglobulin cytoplasmic domain. The genes and/or cDNAs for the heavy and light chain corresponding to the desired hybridomas are isolated and modified if desired. Modifications include humanization, point mutations, deletions, additions that yield improved properties such as changes in affinity or ease of expression. Once the genes and/or cDNAs for the immunoglobulins are isolated, humanization can take place through the genetic engineering of the sequences encoding the MAbs. Humanization is an option, but may not be necessary for short term treatments. Furthermore, light and heavy chains from different antibodies can be introduced into a single cell to modulate the properties of the new surface antibodies.

Various procedures known in the art may be used for the production of antibodies to a target antigen. For the production of antibody, various host animals can be immunized by injection with the target antigen, or a derivative (*e.g.*, fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the target antigen or fragment thereof can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet

hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies using B cells generated by the foregoing immunization methods, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein [*Nature* **256**:495-497 (1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor *et al.*, *Immunology Today* **4**:72 1983); Cote *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**:2026-2030 (1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)]. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology [PCT/US90/02545]. According to the invention, techniques described for the production of single chain antibodies [U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted to produce target antigen-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries [Huse *et al.*, *Science* **246**:1275-1281 (1989)] to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for an target antigen, or its derivatives, or analogs.

It is possible to introduce the heavy and light chain as a single chain antibody construct (SCA). In this case, the intracellular domain will be coupled to signal transduction in two ways. In one case, the SCA will be coupled to both Hu-IFN-γR1 and Hu-IFN-γR2 intracellular domains; both constructs will be co-infected into cells. Intracellular domains of other receptors that require the aggregation of two JAK kinases and subsequent STAT proteins can also be used. In the second case, the intracellular domain will be chosen from among single chain receptors such as EGF and EPO. The this construct only a single chain needs to be expressed in the cells by infecting with only one r troviral construct.

In the production of antibodies, screening for the desired antibody can be

accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of an target antigen, one may assay generated hybridomas for a product which binds to an target antigen fragment containing such epitope. For selection of an antibody specific to an target antigen from a particular species of animal, one can select on the basis of positive binding with target antigen expressed by or isolated from cells of that species of animal.

Cytokine and Growth Factor Receptor Extracellular Domains

As described above, the ligand binding portion of a cytokine or growth factor receptor extracellular domain can be used as the extracellular domain of a chimeric receptor of the invention. Examples of cytokine and growth factor receptors are described above. The methods for isolating the extracellular domains are routine in the art, such as PCR amplification of the region coding for such receptor, specific endonuclease cleavage (perhaps after introduction of the endonuclease cleavage site by site directed mutagenesis or other techniques), and gene synthesis. The molecular biological manipulations are described in greater detail, *infra*, and are exemplified in the Examples.

Cytokine and Growth Factor Extracellular Domains

A particularly interesting feature of the present invention is that is allows targeting of cells that express a chimeric receptor to cells that express a

cytokine or growth factor rec ptor using the corresponding cytokine or growth factor. This aspect of the inv ntion advantageously provides for highly specific targeting, which is not dependent on generating a receptor-specific antibody. The cytoplasmic domain of a chimeric receptor of this aspect of the invention may be generated from the full length cytokine or growth factor, or the receptor binding fragment thereof. Examples of cytokines and growth factors suitable for use according to the invention are provided *supra*.

Genetically Engineered Genes for Chimeric Receptors

Preparation of Expression Vectors for Synthetic Receptors. The chimeric receptor consists of two parts: the ectodomain targeting a specific antigen or hapten; the intracellular domain of the specific cytokine (*e.g.*, interferon), growth factor, or other ligand. Furthermore, combinations of the cytoplasmic domain from one cytokine can be mixed with that from another. The synthetic cell surface receptor provides the targeting domain; the internal domains provide the polypeptides that activate the desired signal transduction mechanisms. By using intracellular domains from different cytokines, novel new signal transduction mechanisms can be obtained. In one embodiment, the first chain (ligand-binding; Hu-IFN-γR1) and second (accessory factor 1, AF-1; Hu-IFN-γR2) chain of the human IFN-γR complex as the intracellular components are used. A variety of retroviral vectors or simple plasmid vectors can be used for these purposes as well as many other eukaryotic viral and non-viral based vectors. The requirement for useable final constructs is that the synthetic receptor chains are expressed on the cell surface of transfected or infected cells.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (h rein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal*

Cell Culture [R.I. Fr shney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (*e.g.*, plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, *i.e.*, capable of replication under its own control.

A "cassette" refers to a segment of DNA that can be inserted into a vector at specific restriction sites. The segment of DNA encodes a polypeptide of interest, and the cassette and restriction sites are designed to ensure insertion of the cassette in the proper reading frame for transcription and translation. Cassettes of the invention may provide an insertion site for DNA encoding an extracellular domain in reading frame with a coding region for the transmembrane domain and a cytoplasmic domain; an insertion site for a DNA encoding an extracellular domain and transmembrane domain in reading frame with a cytoplasmic domain; a coding region for an extracellular domain and a transmembrane domain with an insertion site in reading frame for a cytoplasmic domain; or a coding region for an extracellular domain with a cassette insertion site in reading frame for a transmembrane domain and a cytoplasmic domain. In the first two cassette constructs, a specific cell signal pathway can be used with a multiplicity of ligand/binding partner specificities. In the second pair of cassette constructions, the ligand/binding partner specificity will be constant, with different intracellular signal pathways employed. In still another mbodiment, a cassett ins rtion site can rec ive a construct ncoding the extracellular-transmembrane-cytoplasmic domains. In still an ther embodiment, two cass tte insertion sites can be provided: one 5′ to a transmembrane coding region for DNA encoding an extracellular domain; the other 3′ to the

transmembrane coding region for DNA encoding the cytoplasmic domain. The cassette vector may include a signal sequence (see *infra*) 5′ to the site for the extracellular domain, or a signal sequence may be inserted. It should be noted that the arrangement of coding regions for specific domains will ordinarily be 5′-signal sequence-extracellular-transmembrane-cytoplasmic-3′, which will provide for the proper orientation upon expression as discussed below in connection with "signal sequences."

The present invention generally concerns introduction of a nucleic acid molecule, preferably and expression vector, into a cell, whether by transfection, transduction, or transformation (which terms are frequently, if improperly, used interchangeably in the art). A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. A cells has been "transduced" by heterologous DNA when the DNA recombinantly inserts in a chromosome. Preferably, the transforming DNA should be integrated (covalently linked) into chromosomal DNA making up the genome of the cell, *i.e.*, transduced in the cell.

"Heterologous" DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. According to the invention, DNA encoding a chimeric receptor, which does not naturally occur in the cell, is heterologous DNA. Another common synonym for heterologous DNA is "exogenous" DNA.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester anologs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes

double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook *et al.*, *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more imp rtant, and the length of the oligonucleotide determines its specificity (*see* Sambrook *et al.*, *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least

about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

"Homologous recombination" refers to the insertion of a foreign DNA sequence of a vector in a chromosome. Preferably, the vector targets a specific chromosomal site for homologous recombination. For specific homologous recombination, the vector will contain sufficiently long regions of homology to sequences of the chromosome to allow complementary binding and incorporation of the vector into the chromosome. Longer regions of homology, and greater degrees of sequence similarity, may increase the efficiency of homologous recombination.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purpos s of defining the present invention, the promoter sequ nce is bounded at its 3' terminus by the transcription initiation sit and extends upstream (5' dir ction) to include the minimum number of bases or lements necessary to initiate transcription at levels det ctable abov

background. Within th promoter sequ nce will b found a transcription initiation site (conv niently defin d for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control of," "operably associated with," or "operationally associated with" transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

A "signal sequence" is included at the beginning (5') of the coding sequence of a protein to be expressed on the surface of a cell or secreted for a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms. According to the invention, an expression vector providing for expression of a chimeric receptor will include a signal sequence 5' to the region coding for the extracellular domain, in reading frame therewith. Usually, the signal peptide encoded by the signal sequence is cleaved upon translation of the mRNA and translocation of the expressed protein, resulting in the mature protein.

As used herein, the term "sequence homology" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck *et al.*, 1987, Cell 50:667).

Accordingly, the term "sequence similarity" in all its grammatical forms r fers to the degr e of identity or correspondence between nucleic acid or amino acid sequenc s of proteins that do not share a common evolutionary origin (*see*

Reeck *et al., supra*). However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and not a common evolutionary origin.

In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 50% (preferably at least about 75%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis *et al., supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 30% of the amino acids are identical, or greater than about 60% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

Genes encoding a chimeric receptor, can be prepared using standard genetic engineering techniques, using starting materials (the extracellular domain and cytoplasmic domain) isolated from any source, particularly from a human cDNA or genomic library. Methods for obtaining such genes are well known in the art, as described above (*see, e.g.*, Sambrook *et al.*, 1989, *supra*), as are the sources for the native genes or recombinantly derived versions thereof. Accordingly, any

animal cell potentially can serve as the nucleic acid sourc for the molecular cloning of these genes. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), and preferably is obtained from a cDNA library prepared from tissues with high level expression of the protein, by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell (See, for example, Sambrook *et al.*, 1989, *supra*; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

The present invention also relates to genes encoding analogs and derivatives of the extracellular domains and cytoplasmic domains of the invention, that have the same or homologous functional activity as the native proteins, and homologs thereof from other species. The production and use of derivatives and analogs related to these protein components of a functional chimeric receptor are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities, such as but not limited to, activation of a Jak kinase or Stat protein.

Derivatives of the extracellular domain or cytoplasmic domain can be made by altering encoding nucleic acid sequences of the component proteins by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity relative to native receptor molecules.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as native genes for xtracellular and cytoplasmic domains may be used in the practice of th present inventi n. Thes includ but are not limited to allelic genes, homologous genes from other sp cies, and nucleotide sequences comprising all or portions of the genes which are altered by the substitution of different codons that encode the

same amino acid residue within the sequence, thus producing a silent change. Altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution, are contemplated. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH_2 can be maintained.

Other preferred substitutions are with divergent amino acid residues in homologous proteins from other species.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (*i.e.*, His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β-turns in the protein's structure.

Additionally, a nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Indeed, such mutations are required to create the chimeric receptor encoding nucleic acid constructs of the invention. Preferably, such mutations enhance the functional activity of the mutated gene product. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., *et al.*, 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant *et al.*, 1986, Gene 44:177; Hutchinson *et al.*, 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

Using the DNA encoding extracellular and cytoplasmic domains selected for the chimeric receptor, the specific domains are isolated as described above and joined together using standard genetic engineering techniques. Examples of these techniques are provided in the Examples, *infra*. Generally, the desired region for the extracellular domain or the cytoplasmic domain is amplified using PCR, cloned, and inserted in an expression vector construct in correct reading frame with other components of the chimeric receptor. PCR, endonuclease cleavage, and insertion of DNA in vectors are all well established, routine techniques that require no further elaboration here.

The gene encoding a chimeric receptor can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a

cloning vector which has compl mentary cohesive termini. Howev r, if th complementary restriction sites used to fragment the DNA ar not pr sent in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences form the yeast 2μ plasmid. Restriction endonuclease sites may be created in the cloning vector to provide for easy excision of the coding sequence for insertion in an expression vector.

The nucleic acid, preferably DNA, molecules coding for the chimeric receptor can be inserted into one or more appropriate expression vector(s), *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acids encoding a chimeric receptor of the invention are operationally associated with a promoter in an expression vector or vectors of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding the extrac llular domain, the cytoplasmic domain, and/or its flanking regi ns. Alternatively, a h t rologous promoter, preferably one that enhances expr ssion in the host cell, is used.

The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

Genetically Engineered Cells That Express a Chimeric Receptor

According to the invention, an expression vector or vectors that provide for expression of one or more chimeric receptor(s) are introduced into cells of an animal, preferably mammal, *e.g.*, by transfection *in vitro* or *in vitro*. Such cells can be used *in vitro* for identification of agonists and antagonists the extracellular domain of the chimeric receptor, where cellular activation will not proceed with the native receptor having that extracellular domain.

The cell into which the recombinant vector or vectors comprising the nucleic acid encoding the chimeric receptor is cultured in an appropriate cell culture medium under conditions that provide for expression of the chimeric receptor and formation of a functional chimeric receptor complex by the cell. In a specific embodiment, *infra*, the cell is an African Green Monkey kidney COS-1 cell. Other appropriate cells include, but are not limited to, chinese hamster ovary (CHO) cells; RI.I, B-W and L-M cells; other African Green Monkey kidney cells (*e.g.*, COS 7, BSC1, BSC40, and BMT10); murine fibroblast NIH-3T3 cells; and human cell lines such as HeLa, 2fTGH cells, HEp-2, and human kidney 293 cells; and other cells routinely used in *in vitro* cultures, *i.e.*, for which optimal culture conditions have been established. In an alternative embodiment, the present invention further provides for introducing the recombinant vector or vectors that provide for expression of a functional chimeric receptor complex into primary cells that either lack a functional receptor complex, or lack expression of a particular species of a functional Jak or Stat protein.

Any of the methods for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of a chimeric receptor may be c ntrolled by any promot r/enhancer element kn wn in the art, but these regulatory elem nts must be functional in the host selected for expression. Promoters which may be used to control gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42); and animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic and are useful for gene therapy applications: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378). In a specific embodiment, *infra*, the genes encoding both IL-10R chain and CRFB4 are under control of the powerful promoter of human elongation factor 1α [Mizushima and Nagata, *Nucl.*

Acids Res. **18**:5322 (1990)].

Mammalian expression vectors contemplated for use in the invention include vectors with inducible promoters, such as the dihydrofolate reductase (DHFR) promoter, *e.g.*, any expression vector with a *DHFR* expression vector, or a *DHFR*/methotrexate co-amplification vector, such as pED (*Pst*I, *Sal*I, *Sba*I, *Sma*I, and *Eco*RI cloning site, with the vector expressing both the cloned gene and *DHFR*; *see* Kaufman, *Current Protocols in Molecular Biology*, 16:12 (1991). Alternatively, a glutamine synthetase/methionine sulfoximine co-amplification vector, such as pEE14 (*Hind*III, *Xba*I, *Sma*I, *Sba*I, *Eco*RI, and *Bcl*I cloning site, in which the vector expresses glutamine synthase and the cloned gene; Celltech). In another embodiment, a vector that directs episomal expression under control of Epstein Barr Virus (EBV) can be used, such as pREP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive RSV-LTR promoter, hygromycin selectable marker; Invitrogen), pCEP4 (*Bam*H1, *Sfi*I, *Xho*I, *Not*I, *Nhe*I, *Hind*III, *Nhe*I, *Pvu*II, and *Kpn*I cloning site, constitutive hCMV immediate early gene, hygromycin selectable marker; Invitrogen), pMEP4 (*Kpn*I, *Pvu*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, *Bam*H1 cloning site, inducible methallothionein IIa gene promoter, hygromycin selectable marker: Invitrogen), pREP8 (*Bam*H1, *Xho*I, *Not*I, *Hind*III, *Nhe*I, and *Kpn*I cloning site, RSV-LTR promoter, histidinol selectable marker; Invitrogen), pREP9 (*Kpn*I, *Nhe*I, *Hind*III, *Not*I, *Xho*I, *Sfi*I, and BamHI cloning site, RSV-LTR promoter, G418 selectable marker; Invitrogen), and pEBVHis (RSV-LTR promoter, hygromycin selectable marker, N-terminal peptide purifiable via ProBond resin and cleaved by enterokinase; Invitrogen). Selectable mammalian expression vectors for use in the invention include pRc/CMV (*Hind*III, *Bst*XI, *Not*I, *Sba*I, and *Apa*I cloning site, G418 selection; Invitrogen), pRc/RSV (*Hind*III, *Spe*I, *Bst*XI, *Not*I, *Xba*I cloning site, G418 selection; Invitrogen), and others. Vaccinia virus mammalian expression vectors (*see*, Kaufman, 1991, *supra*) for use according to the invention include but are not limited to pSC11 (*Sma*I cloning site, TK- and β-gal selection), pMJ601 (*Sal*I, *Sma*I, *Afl*II, *Nar*I, *Bsp*MII, *Bam*HI, *Apa*I, *Nhe*I, *Sac*II, *Kpn*I, and *Hind*III cloning site; TK- and β-gal selection), and pTKgptF1S (*Eco*RI, *Pst*I, *Sal*I, *Acc*I, *Hind*II, *Sba*I, *Bam*HI, and Hpa cloning site, TK or XPRT selection).

In a specific embodiment, a phosphorylatable form of the chimeric receptor can be expressed [*See* European Patent No. 0372707 by Sidney Pestka], *e.g.*, by using plasmid pGEX-TTK (Pharmacia, product no. 27-45-8701).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, cleavage [*e.g.*, of signal sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. Expression in eukaryotic cells can increase the likelihood of "native" glycosylation and folding of a heterologous protein. Moreover, expression in mammalian cells can provide a tool for evaluating chimeric receptor activity. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu *et al.*, 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990).

Gene Therapy and Transgenic Vectors

In one embodiment, a gene encoding a chimeric receptor is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, adipose tissue can

be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt *et al.*, *Molec. Cell. Neurosci.* 2:320-330 (1991)], an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.* [*J. Clin. Invest.* 90:626-630 (1992)], and a defective adeno-associated virus vector [Samulski *et al.*, *J. Virol.* 61:3096-3101 (1987); Samulski *et al.*, *J. Virol.* 63:3822-3828 (1989)].

Preferably, for *in vivo* administration, an appropriate immunosuppressive treatment is employed in conjunction with the viral vector, *e.g.*, adenovirus vector, to avoid immuno-deactivation of the viral vector and transfected cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon-γ (IFN-γ), interleukin-10 (IL-10), or anti-CD4 antibody, can be administered to block humoral or cellular immune responses to the viral vectors [*see, e.g.*, Wilson, *Nature Medicine* (1995)]. In a further embodiment, the vector can be engineered to express such immunosuppressive cytokines. In addition, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson *et al.*, U.S. Patent No. 5,399,346; Mann *et al.*, *Cell* 33:153 (1983); Temin *et al.*, U.S. Patent No. 4,650,764; Temin *et al.*, U.S. Patent No. 4,980,289; Markowitz *et al.*, *J. Virol.* 62:1120 (1988); Temin *et al.*, U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty *et al.*; and Kuo *et al.*, *Blood* 82:845 (1993).

Targeted gene delivery is described in International Patent Publication WO 95/28494, published October 1995.

Alternatively, the vector can be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a

marker [Felgner, et. al., *Proc. Natl. Acad. Sci. U.S.A.* **84**:7413-7417 (1987); *see* Mack y, *et al., Proc. Natl. Acad. Sci. U.S.A.* **85**:8027-8031 (1988)]. The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes [Felgner and Ringold, *Science* **337**:387-388 (1989)]. The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting [*see* Mackey, et. al., *supra*]. Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter [*see, e.g.*, Wu *et al., J. Biol. Chem.* **267**:963-967 (1992); Wu and Wu, *J. Biol. Chem.* **263**:14621-14624 (1988); Hartmut *et al.*, Canadian Patent Application No. 2,012,311, filed March 15, 1990].

In Vitro Assay Systems

As discussed above, and exemplified below (Examples 1 and 3), the chimeric receptors of the present invention are valuable tools for probing cellular responses to cytokines, growth factors, and other molecules by dissecting the signal transduction components of the receptor; by identifying the specificity or degeneracy of different Jak and Stat activation pathways; and by developing new cell targets for drug discovery programs by allowing for activation of a particular, high s nsitivity signal transduction pathway with any ligand or binding partn r of inter st.

Thus, in a particular aspect, the present invention advantageously provides for

identification of an agonist or antagonist for a particular growth factor or cytokine, using routine screening techniques and a highly sensitiv assay c ll line that expresses a chimeric receptor comprising an extracellular domain specific for the ligand for which an analog (agonsit or antagonist) is of interest, and a cytoplasmic domain that provides for strong signal transduction in the cell. In a specific embodiment, *infra*, CHO cells are modified to be responsive to IL-10.

Thus, expression of a functional chimeric receptor in cells engineered to indicate the activity of the ligand for the extracellular domain of the receptor expressed after transfection or transduction of the cells is possible. Accordingly, the present invention contemplates a method for identifying specific ligands, and more particularly ligands that either agonize or antagonize receptor activity, using various screening assays known in the art.

Any screening technique known in the art can be used to screen for agonists or antagonists. The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to and agonize or antagonize the chimeric receptor *in vivo*. For example, natural products libraries can be screened using assays of the invention for molecules that agonize or antagonize the natural ligand activity. In addition, screening for anti-idiotypic antibodies, *e.g.*, based on competitive binding with the natural ligand for the chimeric receptor can be used to identify anti-idiotypic agonists and antagonists.

Another approach to screening for biologically active compounds uses recombinant bacteriophage to produce large libraries. Using the "phage method" [Scott and Smith, 1990, *Science* **249**:386-390 (1990); Cwirla, *et al.*, *Proc. Natl. Acad. Sci.*, **87**:6378-6382 (1990); Devlin *et al.*, *Science*, **249**:404-406 (1990)], very large libraries can be constructed (10^6-10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method [Geysen *et al.*, *Molecular Immunology* **23**:709-715 (1986); Geysen *et al. J. Immunologic Method* **102**:259-274 (1987)] and the method of Fodor *et al.* [*Science* **251**:767-773 (1991)] are examples. Furka *et al.* [*14th International*

Congress of Biochemistry, Volume 5, Abstract FR:013 (1988); Furka, *Int. J. Peptide Protein Res.* 37:487-493 (1991)], Houghton [U.S. Patent No. 4,631,211, issued December 1986] and Rutter *et al.* [U.S. Patent No. 5,010,175, issued April 23, 1991] describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In another aspect, synthetic libraries [Needels *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10700-4 (1993); Ohlmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993); Lam *et al.*, International Patent Publication No. WO 92/00252; Kocis *et al.*, International Patent Publication No. WO 9428028, each of which is incorporated herein by reference in its entirety], and the like can be used to screen for IL-10 receptor ligands according to the present invention.

Various assays may be performed to determine whether the candidate molecule, *e.g.*, prepared in a combinatorial library as described above, or found in a library of natural products, acts as an agonist or antagonist of the natural ligand for the extracellular domain of a chimeric receptor. In a preferred embodiment, these tests employ recombinant cells engineered to express a functional chimeric receptor complex. In a specific embodiment, an assay of the invention employs COS-1 or CHO cells transfected with vectors that provide for expression of a functional chimeric receptor.

Detection of chimeric receptor-mediated activation (or inhibition of activation) can be accomplished by evaluating changes in cell targets, as described above. Chimeric receptor-mediated activation can be detected and quantified (or semi-quantified) by detecting increased phosphorylation of a Jak protein, such as Tyk2 in the case of the CRFB4 cytoplasmic domain, activation of a Stat protein, such as Stat1 or Stat3 in the case of the CRFB4 cytoplasmic domain, decreased production of a responsive molecule, *e.g.*, interferon-γ, etc.

As shown in a specific embodiment, *infra*, phosphorylation of Tyk2 can be measured by double immunoprecipitation assays with an anti-Tyk2 antibody and an anti-phosphoprotein antibody, and detection of an identical band in both precipitations. Alternatively, Tyk2 phosphorylation can be evaluated by co-

transfection of cells transfected with a chimeric receptor that comprises the CRFB4 cytoplasmic domain with a vector for expression of Tyk2, and detection of phosphorylation by gel shift, antibody probes, or radiolabelling with ^{32}P. These specific examples are clearly generalizable to all of the signal transduction domains from cytokine or growth factor receptors.

Activation of Stat proteins can be evaluated by any of the techniques described in International Patent Publication No. WO 95/08629, by Darnell *et al.*, published March 30, 1995, and International Patent Publication No. WO 93/19179, by Darnell *et al.*, published September 30, 1993. In specific embodiments, Stat protein activation can be evaluated by detecting Stat1 dimerization, or in gel-shift electrophoretic mobility shift assays (EMSA) using binding sites. For example, a 22 base pari sequence containing a Stat1α binding site corresponding to the GAS element in the promoter region of the human IRF-1 gene can be used to evaluate Stat1α activation [*see* Kotenko *et al.*, *J. Biol. Chem.* **270**:20915-21 (1995)].

Interferon-γ expression can be detected using biological assays, *e.g.*, antiviral assays, or immunometrically, such as by ELISA, ELISPOT, immunopreciptiation, etc.

Therapeutics

The present invention provides a unique opportunity to enhance immune responses, to control inflammatory or autoimmune responses, and to treat diseases or disorders that result from a deficiency of an intracellular signaling pathway by circumventing that pathway.

Targeting effector cells. As described in detail below, either antibody molecules or a cytokine or growth factor can be used to target effector cells to target cells. Such target cells may be tumor cells, virally infected cells, bacterially infected cells, autoimmun cells, or inflammatory cells. Various strategies can be used to target and effector cell to the target cell. The effector cells can be used to control cell growth, *e.g.*, by mediating cell killing, where disregulation of growth or activity is involved in a disease or disorder. "Disregulation" refers to a

condition of unregulated growth or activity, such as is found in autoimmune response, pathological inflammatory response, and tumors.

The present invention is directed the treatment of tumors, particularly solid tumors. Examples of solid tumors that can be treated according to the invention include sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

In another embodiment, dysproliferative changes (such as metaplasias and dysplasias) are treated or prevented in epithelial tissues such as those in the cervix, esophagus, and lung. Thus, the present invention provides for treatment of conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-79). Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or rgan, without significant alteration in structure or function. As but one xample, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one typ of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can

occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder. For a review of such disorders, see Fishman *et al.*, 1985, *Medicine*, 2d Ed., J. B. Lippincott Co., Philadelphia.

The present invention is also directed to treatment of non-malignant tumors and other disorders involving inappropriate cell or tissue growth by administering a therapeutically effective amount of an effector cell of the invention. The invention may be used to treat psoriasis, a dermatologic condition that is characterized by inflammation and vascular proliferation; benign prostatic hypertrophy, a condition associated with inflammation and possibly vascular proliferation; and cutaneous fungal infections. Treatment of other hyperproliferative disorders is also contemplated.

Autoimmune diseases include immune complex-induced vasculitis [Cochrane, 1984, *Springer Seminar Immunopathol.* 7:263 (1984)], glomerulonephritis [Couser *et al.*, *Kidney Inst.* **29**:879 (1985)], hemolytic anemia [Schreiber and Frank, *J. Clin. Invest.* **51**:575 (1972)], myasthenia gravis [Lennon, *et al.*, *J. Exp. Med.* **147**:973 (1978); Biesecker and Gomez, *J. Immunol.* **142**:2654 (1989)], type II collagen-induced arthritis [Watson and Townes, *J. Exp. Med.* 162:1878 (1985)]; experimental allergic and hyperacute xenograft rejection [Knechtle, *et al.*, J. Heart Transplant 4(5):541 (1985); Guttman, Transplantation 17:383 (1974); Adachi, *et al.*, Trans. Proc. 19(1):1145 (1987)]; rheumatoid arthritis, and systemic lupus erythematosus (SLE).

An antibody generated to an antigen on a target cell can be used to provide a membrane Ig/membrane SCA extracellular domain of an effector cell. Similarly, an cytokine or growth factor corresponding to a receptor found in reasonably

high density on the target cell can be used to target the effector cell.

The cytoplasmic domain of the effector cell is selected for high level activation of the cell upon ligand binding, and will be readily correlated depending on the effector cell chosen. For example, if the effector cell is a cytotoxic T cell, the cytoplasmic domain may be obtained from the IL-2 receptor. If the effector cell is an inflammatory cell (phagocyte), the cytoplasmic domain may be obtained from the TNF-α receptor or IFN-α receptor. When the cells chosen for infection are CD8 cytotoxic T-cells or NK cells, the use of the Hu-IFN-γR1 and Hu-IFN-γR2 chains can be used to stimulate the cell as would be provided by Hu-IFN-γ when the synthetic receptor is activated.

Suitable antigens for targeting the chimeric mIg/R receptor include tumor associated antigen (TAA), viral antigen, growth factor receptors expressed at high levels on tumor cells, bacterial antigens (*e.g.*, leishmania, mycobacterium, etc.), or receptor molecules characteristic of a tumor, such as IL-2 on certain lymphomas. Any of the foregoing receptors can be used as targets for chimeric receptors that employ a cytokine or growth factor extracellular domain.

Various specificities of effector cells and target cells are listed below:

Extracytoplasmic domain on effector cell	Target on target cell (cell specificity or disease)
TNF	TNF-receptor (inflammatory cell; sepsis)
IL-2	IL-2R (lymphoma; autoantigen-specific T lymphocyte)
IL-1	IL-1R (inflammatory cell; sepsis)
IL-4	IL-4R (B cell lymphoma)
NGF	NGF-R (neuroblastoma)
TGF	TGF-R (tumor cell)

The foregoing effector/target combinations are provided purely as examples;

many more ff ctor/target combinations are known in the art, and can be readily adapted with the chimeric receptors of the present invention to provide for therapy of the foregoing disease conditions.

Preferably, autologous effector cells are obtained from the subject in need of treatment for introduction of an expression vector for a chimeric receptor of the invention. In another embodiment of the procedure, instead of using freshly prepared cells form a human donor, it is possible to use cytotoxic cell lines that could be transfected or infected with various expression vectors expressing the receptor chains. These could be cytotoxic T-cells, NK cells, macrophages, or other cytotoxic cells. The cells with the synthetic receptor could be maintained in culture and used to target cancers in patients. The cells, in this case, would be heterologous cells that would eventually be eliminated by the host. This would be an internal intrinsic safety procedure. With the use of multiple cell lines developed to express antibodies or other molecules that could target the specific cancer cells, a panorama of cells would be available in cultures to use for many different cancers. Although antibodies would be a major targeting construct, other molecules that could target the surface of cancer cells could be used. For example, the expression of a transmembrane protein expressing IL-2 extracellularly, but attached to the transmembrane and intracellular domain of the activating molecules (*i.e.*, IL-2/γR1 and IL-2/γR2 chimeric constructs) could be used to eliminate T-cell leukemia cells. The T-cell receptor itself could be used as an ectodomain coupled to different intracellular domains. The present invention provides a recognition chain on the surface of the cytotoxic cell attached to the activating intracellular domains. On activation of the complex by attachment to the target cell, the cytotoxic cell would be activated to kill the target cells. The use of autologous cells could be coupled by leucapheresis, isolation of particular cytotoxic cells, retroviral gene transfer, and return to the patient. Alternatively, a battery of cytotoxic heterologous cells with the various synthetic receptors or ligands on the cell surface could be available for use as necessary.

Enhancement of Targeting of Synthetic Receptors of Tumor Cells. Interferons have been shown to enhance the expression of tumor associated antigens

effectively in cancers (REFS). By treating patients with an interferon (IFN-α, IFN-ß, IFN-γ, IFN-ω and/or IFN-τ) prior to intr duction of the cytotoxic cell carrying the synthetic receptors, the cytotoxic cells will have a greater number of target cells that are positive for the expression of the tumor associated antigen. This preliminary treatment addresses one of the major problems in cancer therapy, namely, the heterogeneity of tumor cells. By first treating the patients with an interferon, virtually all the tumor cells will be expressing the TAA rather than a small percentage of the population.

Reconstitution of cytokine or growth factor sensitivity. In some conditions, the intrinsic or normal endogenous pathway for cellular activation is blocked. The examples, *infra*, surprisingly show that cellular activation can be achieved by alternative routes. Thus, if a disease or disorder is associated with a deficiency of a particular Jak or Stat protein, the present invention provides for circumventing that deficiency by providing a chimeric receptor with the extracellular domain from the native receptor that is non-functional due to the deficiency and a cytoplasmic domain that activates a different Jak or Stat protein. In a specific example, a chimeric receptor may also be used in gene therapy where patients lack one of the Jak kinases. Specifically, patients lacking Jak3 have a SCID phenotype that would be corrected with chimeric receptors consisting of the IL-2 receptor γ_c chain extracellular domain and an intracellular domain that would recruit Tyk2, Jak1, or Jak2, *e.g.*, CRFB4.

Transfection and Infection of Cells. Plasmids can be transfected into animal cells by a large number of procedures, as described above in the section relating to gene therapy. In the case of retroviral vectors, high titer stocks of viruses are prepared for infection of cells. In a preferred embodiment of high efficiency transfer of the vector to cells, the procedures of Dougherty and Ron [International Patent Publication No. WO 95/07358, published March 16, 1995, by Dougherty *et al.*; and Kuo *et al.*, *Blood* 82:845 (1993)] are employed. As describ d above, all this is accomplished without the treatment f the patient with th cytokine itself, thus avoiding side effects mediat d by the cytokine, but by the introduction of the synthetic receptor ectodomain coupled to the intracellular domain that controls the signal transduction pathway to be

activated into the cells. As noted above, a wide variety of combinations are possible and even multiple signals can be generated by preparing a set of retroviral vectors with different intracellular domains. Since at high multiplicity, several retroviral vectors can infect and integrate into each cell, multiple pathways that activate the cytotoxic signals can be initiated on binding of the synthetic receptor to the target cell.

It is possible to remove cells expressing the antibody receptor in cases where more cells than desirable are present. The cells can be removed by *ex vivo* or *in vivo* procedures. For *ex vivo* procedures, the peripheral blood leukocytes are removed by leucapheresis and passed through membranes, netting, filters, tubes, columns, or other matrices containing bound antigen. The cells binding antigen are removed and the remaining cells returned to the host. To remove cells *in vivo*, a soluble dimer or higher oligomer of the antigen is administered intravenously to binding to the cells. Upon binding the cells are activated and will self-destruct. Another safety backup is the simple blocking of the receptor without activation. This can be done by the intravenous administration of soluble antigen monomer. Binding of the monomer to the synthetic receptor on the cell surface will block interaction of the receptor with cells expressing the antigen and thus prevent dimerization of the receptor and consequent activation of the cells. The soluble monomer binding to the synthetic cell surface receptor can therefore be used to modulate the response if desirable.

EXAMPLES

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention.

EXAMPLE 1: Signalling By IFN-γ With Chimeric IFN-γR2 Chains Containing Intracellular Domains From Various Receptors

To assess whether the specificity of signal transduction resides in the Jaks, a variety of chimeric receptors based on the human IFN-γ receptor complex were prepared. The extracellular domain of the second human IFN-γ receptor chain,

designated Hu-IFN-γR2 or AF-1 [Soh *et al., J. Biol.. Chem.*, **269**:18102-18110 (1994)], was fus d to the transmembrane and intracellular domains of vari us receptors. Notably, this receptor was fused to a signal transduction chain of previously unknown function. The present Example provides evidence that this cytokine signal transduction chain of unknown function is the signal transduction chain for the IL-10 receptor.

Materials and Methods

Reagents, Restriction Endonucleases and Other Enzymes. *Taq* polymerase and all restriction endonucleases were from Boehringer Mannheim Biochemicals or New England Biolabs; Sequenase 2.0 and T4 DNA ligase were from United States Biochemical Corporation. The [α-^{32}P]dATP and [γ-^{32}P]ATP were from New England Nuclear. The crosslinker bis(sulfosuccinimidyl)suberate (BS^3) was from Pierce Chemical Co. All other chemical reagents were analytical grade and purchased from United States Biochemical Corp.

Plasmid Construction. The vector pγR2 expressing the Hu-IFN-γR2 chain under control of CMV promoter was constructed as described previously [Soh *et al., Cell*, **76**:793-802 (1994); Kotenko *et al., J. Biol. Chem.*, **270**:20915-20921 (1995)]. The vector pγR1 expressing the Hu-IFN-γR1 chain was constructed as follows. The pHu-IFN-γR8 plasmid [Kumar *et al., J. Biol. Chem.*, **264**:17939-17946 (1989)] was digested with *Hind*III restriction endonuclease, incubated with the large fragment of DNA polymerase I and dNTPs to fill in the ends and then digested with *Bam*HI restriction endonuclease. The fragment containing the Hu-IFN-γR1 cDNA was ligated into *Eco*RV and *Bam*HI sites of the pcDNA3 vector (Invitrogen).

The Hu-IFN-αR1 cDNA was recloned from HuIFNAR1/pcDNAI [Lim, Ph.D. Thesis, Rutgers University, Piscataway, NJ, (1995)] into pcDNA3 with *Eco*RI and *Xba*I restriction endonucleases. The expression vector was designated pαR1. The PCR products for Hu-IFN-αR2 cDNA were obtained by a nested PCR proc dure with a λpCEV15 phage DNA mixture isolated from about 5 x 10^6 phage clones from the human M426 cell library [Miki *et al., Gene*, **83**:137-146 (1989)] as template. The primers for the first round of PCR w r :

5'-GCCGCAAGGCGAGAGCTGC-3' (SEQ ID NO:5) specific for the 5' end of Hu-IFN-αR2 cDNA [Novick *et al.*, *Cell*, **77**:391-400 (1995)]; and λpCEV15 vector primer 5'-AGATCTAAGCTTGGCCGAGG-3' (SEQ ID NO:6). For the second round the same 5' primer and primer 5'- GCGGAATT**CTTAATCACTGGGGCACAG** -3' (SEQ ID NO:7) specific for the 3' end (bases 1204 - 1222) of Hu-IFN-αR2 cDNA (boldface) containing an *Eco*RI site within the primer were used. The PCR product was digested with *Eco*RI restriction endonuclease and ligated into *Eco*RI and blunt ended *Bam*HI sites of the pcDNA3 vector. The expression vector was designated pαR2.

To isolate the Hu-CRFB4 cDNA an oligonucleotide 5'-GTCCATGGCGTGGAGCCTTGGGAG-3' (SEQ ID NO:8) homologous to the 5' end of Hu-CRFB4 cDNA [Lutfalla *et al.*, *Genomics*, **16**:366-373 (1993)] was labeled with [α-^{32}P]dATP with terminal deoxynucleotide transferase to a specific activity of 5 x 10^6 cpm/μg. It was used to screen about 10^6 phages from the human M426 cell library [Miki *et al.*, *supra*]. Two positive clones were purified and plasmids were rescued from these two λ phages. The CRFB4 cDNA was released from one of the rescued plasmids designated pCEV15-CRFB4-1 by digestion with *Tha*I and *Sal*I restriction endonucleases and cloned into *Eco*RV and *Xho*I sites of the pcDNAIneo vector (Invitrogen). The expression vector for the Hu-CRFB4 chain was designated pCRF.

The vector expressing the chimeric receptor Hu-IFN-γR2/Hu-IFN-γR1 (γR2/γR1) was constructed as followed. The asymmetric PCR reaction was performed with a specific primer 5'-GATGCCTCCACTGAGCTTCAGCAACTTTGGATTCCAGTTGTTGC-3' (SEQ ID NO:9) and 100-fold excess of T7 primer with plasmids pHu-IFN-γR8 (the Hu-IFN-γR1 cDNA) and pγR2 as templates in the same reaction mixture. The second round of the PCR was performed with the set of primers 5'-GACCCTCTTTCCCAGCTGC-3' (SEQ ID NO:10) and 5'-GCCACACATCCTCTTTACGC-3' (SEQ ID NO:11) with 1 μl of PCR reaction mixture from the first r und of PCR as template. The final γR2/γR1 PCR product was digest d with *Bst*EII restriction endonuclease and cloned into *Bst*EII and

blunt-ended *Xba*I sites of the pγR2 plasmid. The expression vector was designated pγR2/γR1.

To introduce an *Nhe*I site in the beginning of the transmembrane domain of the Hu-IFN-γR2 cDNA clone, the PCR reaction was performed with two primers 5'-GCCTTTTTTAGTTATTATGTC-3' (SEQ ID NO:12) and 5'-ATCGCTAGCCATTGCTGAAGCTCAGTGGAGG-3' (SEQ ID NO:13) and plasmid pγR2 as a template according to a standard protocol [Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (1989)]. The PCR product was digested with *Bst*XI restriction endonuclease and ligated into *Bst*XI and *Eco*RV sites of the pγR2. The plasmid was designated pγR2*Nhe*I.

To construct chimeras Hu-IFN-γR2/Hu-IFN-αR1, Hu-IFN-γR2/Hu-IFN-αR2, and Hu-IFN-γR2/CRFB4, the PCR reactions were performed with SP6 primer and 5'-GTGGCTAGCTATAGTTGGAATTTGTATTGC-3' (SEQ ID NO:14) or 5'-GTGGCTAGCATAATTACTGTGTTTTTGAT-3' (SEQ ID NO: 15) or 5'-GTGGCTAGCCGTCATCCTCATGGCCTCG-3' (SEQ ID NO:16) primers with plasmids pαR1, pαR2 or pCRF, respectively, as templates. The Hu-IFN-αR1 and Hu-IFN-αR2 PCR products were digested with *Nhe*I and *Apa*I restriction endonucleases and ligated into *Nhe*I and *Apa*I sites of the pγR2*Nhe*I. The CRFB4 PCR product was digested with *Nhe*I and *Xba*I restriction endonucleases and ligated into *Nhe*I and *Xba*I sites of the plasmid pγR2*Nhe*I. The plasmids were designated pγR2/αR1, pγR2/αR2 and pγR2/CRF, respectively.

To create chimera Hu-IFN-γR2/IL-2Rγ_c, the γ_c chain of IL-2R [Takeshita *et al.*, *Science*, **257**:379-382 (1992)] was obtained by RT-PCR as follows. The first strand cDNA synthesis in RT-PCR was performed with poly(dT)$_{18}$ primer with M-MulV reverse transcriptase with total RNA isolated from peripheral blood leukocytes as template. Two PCR rounds were performed. The primers 5'-CGGTTCAGGAACAATCGG-3' (SEQ ID NO:17) and 5'-CAAGCGCCATGTTGAAGCC-3' (SEQ ID NO:18) were used for the first round. For the second round the PCR product from the first round was diluted 100-fold and used as a template for the second round with primers 5'-GTTAGTACCACTTAGGGC-3' (SEQ ID NO:19) and

5'-GTGGCTAGCATGGGAAGCCGTGGTTATC-3' (SEQ ID NO:20). The IL-2Rγ_c PCR product was digest d with *Nhe*I restriction endonuclease and ligated into the *Nhe*I and blunt ended *Xba*I sites of plasmid pγR2*Nhe*I. The resultant expression vector was designated pγR2/γ_c. The nucleotide sequences of the modified regions of all the constructs were verified in their entirety.

Cells, Media and Transfection. The 16-9 hamster x human somatic cell hybrid line is the Chinese hamster ovary cell (CHO-K1) hybrid containing a translocation of the long arm of human Chromosome 6 encoding the HUIFNGR1 (Hu-IFN-γR1) gene and a transfected human HLA-B7 gene [Soh *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**:8737-8741 (1993)]. The 16-9 cells were maintained in F12 (Ham) medium (Sigma) containing 5% heat-inactivated fetal bovine serum (Sigma) (complete F12 medium). HEp-2 cells, a human epidermoid larynx carcinoma cell line, and COS-1 cells, a SV40 transformed fibroblast-like simian cell line, were maintained in DMEM medium (GIBCO) with 10% heat-inactivated fetal bovine serum.

The 16-9 cells were stably transfected with the expression vectors (1-3 μg of super-coiled plasmid DNA per 10^5-10^6 cells) with LipofectAMINE™ Reagent (Life Technologies) according to the manufacturer's instructions for stable transfection of adherent cells. For cotransfection we used 1-3 μg of plasmid DNA with the *neo*^{R} gene and a 10-fold excess of plasmid DNA without *neo*^{R} gene per 10^5-10^6 cells. All cell lines transfected with plasmids carrying the *neo*^{R} gene were selected and maintained in complete F12 medium containing 450 μg/ml of antibiotic G418. COS-1 cells were transiently transfected with the expression vectors by the DEAE-dextran procedure with DMSO shock [Seed *et al.*, *Proc. Natl. Acad. Sci. USA*, **84**:3365-3369 (1987); Sussman *et al.*, *Mol. Cell. Biol.*, **4**:1641-1643 (1984)].

Cytofluorographic Analysis. Cytofluorographic analysis of cells for expression of the HLA-B7 surface antigen was performed as described previously [Jung *et al.*, *Somatic Cell and Molecular Genetics*, **14**:583-592 (1988); Cook *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**:11317-11321 (1992); Hibino *et al.*, *J. Biol. Chem.*, **267**:3741-3749 (1992)]. Hu-IFN-αA/D, a chimeric human int rferon activ on

hamster cells [Rehberg *et al.*, *J. Biol. Chem.*, **257**:11497-11502 (1982)], was used as a control to demonstrat the integrity of th HLA-B7 gene in various cell lines.

Cross-linking of IFN-γ to Receptors. Recombinant Hu-IFN-γ with a specific activity of 2 x 10^7 units/mg was phosphorylated as reported [Rashidbaigi *et al.*, *Proc. Natl. Acad. Sci. USA*, **83**:384-388 (1986); Mariano *et al.*, in *Cytokines: A Practical Approach*, 95-108, (1991)]. The [^{32}P]Hu-IFN-γ was bound to cells and then crosslinked as described previously [Kotenko *et al.*, *supra*].

Antibodies. Rabbit anti-Jak1, anti-Jak2 and anti-Jak3 antibodies were developed against synthetic peptides KTLIEKERFYESRCRPVTPSC (SEQ ID NO:21), DSQRKLQFYEDKHQLPAPKC SEQ ID NO:22) and AKLLPLDKDYYVVREPG (SEQ ID NO:23) corresponding to the end of the kinase-like domains of murine Jak1 and Jak2, and a to sequence within the kinase domain of murine Jak3, respectively. Rabbit anti-Tyk2 antibody was from Santa Cruz Biotechnology (catalog #SC-169). Rabbit anti-Stat1α antibody was raised against the C-terminus of Stat1α. Monoclonal anti-phosphotyrosine antibody was from Sigma (catalog #P3300). Rabbit anti-Hu-IFN-γR2 antibody was prepared with the extracellular domain of Hu-IFN-γR2 as antigen.

Immunoprecipitations, Blottings and Kinase Assay. Cells were starved overnight in serum free media and subsequently stimulated with Hu-IFN-γ (1000 units/ml) for 10 minutes at 37°C. Preparation of cell lysates, immunoprecipitations, blottings and *in vitro* kinase activation assay were performed as described [Kotenko *et al.*, *supra*].

Electrophoretic Mobility Shift Assays (EMSA). EMSAs were performed with a 22 base pair sequence containing a Stat1α binding site corresponding to the GAS element in the promoter region of the human IRF-1 gene (5'-GATCGATTTCCCCGAAATCATG-3') (SEQ ID NO:24) as described [Kotenko *et al.*, *supra*].

Results

Construction of Chimeric Receptors, Expression in COS Cells and Crosslinking. To inv stigate the specific requirements for the intrac llular domain of Hu-IFN-γR2 we created chimeric receptors with the extracellular domain of Hu-IFN-γR2 attached to the transmembrane and intracellular domains of different human receptors as shown in Figure 1. The following receptors were used: two chains of the Hu-IFN-γ receptor complex, Hu-IFN-γR1(γR1) [Aquet *et al.*, *Cell* **55**:273-280 (1988)] or human IFN-γR1t_{456} (γR1t_{456}) the truncated γR1 with the intracellular domain terminated by premature stop codon after amino acid 456 [Cook *et al.*, *supra*], and Hu-IFN-γR2 (γR2) [Soh *et al.*, *Cell*, **76**:793-802 (1994)]; two chains of human IFN-α receptor complex, Hu-IFN-αR1 (αR1) [Uzé *et al.*, *Cell*, **60**:225-234 (1990)] and Hu-IFN-αR2 (αR2) [Novick *et al.*, *supra*]; Hu-CRFB4, a class II cytokine receptor with unknown function [Lutfalla *et al.*, *supra*]; and Hu-IL-2 receptor γ_c chain [Takeshita *et al.*, *supra*]. To confirm that all chimeric receptors can be expressed properly, the ability of expression vectors encoding chimeric receptors to express the proteins was determined. All plasmids were transiently transfected into COS-1 cells and the expression of the receptors were evaluated. The cellular lysates from COS-1 cells transiently transfected with the expression vectors were resolved on SDS-PAGE, transferred to membrane and probed with antibodies to the Hu-IFN-γR2 extracellular domain as all these chimeric receptors contain the Hu-IFN-γR2 extracellular domain. In all cases specific bands were detected (Figure 2). Thus, all vectors encoding the chimeric receptors express these proteins.

It was previously shown that the Hu-IFN-γR2 chain is a part of the human IFN-γ receptor ligand binding complex, although by itself does not bind Hu-IFN-γ; nevertheless, the Hu-IFN-γR2 chain can be detected by crosslinking to Hu-IFN-γ [Kotenko *et al.*, *supra*]. Therefore, crosslinking was used to ascertain that the chimeric receptors were expressed on the cell surface and were able to participate in the human IFN-γ receptor ligand binding complex (Figure 3). All chimeric receptors were stably expressed in 16-9 cells, hamster cells expressing th Hu-IFN-γR1 chain [Soh *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**:8737-8741 (1993)], and antibiotic G418-resistant cell populations were used in crosslinking experim nt. The cell lines w re d signated according to the xtracellular/intracellular domains (*e.g.*, γR2/γR1) of the chimeric receptors

expressed (Figur 1). The crosslinking of labeled IFN-γ to the parental 16-9 c lls r sults in f rmati n of a singl crosslinked band on the SDS-PAGE migrating in the region of 120 kD and corresponding to the Hu-IFN-γ:Hu-IFN-γR1 (IFN-γ:γR1) complex [Kotenko *et al., supra*]. It was shown that the additional band observed in the γR2 cells migrating in the region of 60 kD corresponded to the Hu-IFN-γ:Hu-IFN-γR2 (IFN-γ:γR2) complex [Kotenko *et al., supra*]. In addition to the IFN-γ:γR1 crosslinked complex observed in all cell lines (Figure 3), the mobility of the second band corresponded to the IFN-γ:γR2/X complex (where X represents the transmembrane and intracellular domains of the chimeric receptor expressed) and was different for each chimeric receptor (Figure 3). The IFN-γ:γR2/X complex was observed for all chimeric receptors, indicating that they all were expressed on the cell surface and formed ternary ligand-receptor complexes (γR1:IFN-γ:γR2/X) in all cell lines. Because the Hu-IFN-γ:γR2/γR1 complex has almost the same mobility as the Hu-IFN-γ:γR1 complex formed from the endogenous Hu-IFN-γR1, in γR2/γR1 cells a single crosslinked band in the region of 120 kD (Figure 3, right panels) was observed. In addition, in the 16-9 cells transfected with the γR2/γR1t_{456} (γR2/γR1 chimera, containing a truncated γR1 intracellular domain, γR1t_{456}), the appearance of a faster migrating band representing a cross-linked complex migrating faster than the Hu-IFN-γ:γR2/γR1 complex (Figure 3, right panels) was observed.

Class I MHC Antigen Induction. Class I MHC antigen (HLA-B7 surface antigen) induction was measured to evaluate the ability of the chimeric receptors to support signal transduction upon Hu-IFN-γ treatment in the 16-9 cells transfected with different chimeric receptors. The 16-9 cells, expressing only the Hu-IFN-γR1 chain of the Hu-IFN-γ receptor complex, exhibited little or no response to Hu-IFN-γ (Figure 4A). The 16-9 cells were transfected with an expression vector encoding the intact Hu-IFN-γR2 (γR2) or the chimeric receptors Hu-IFN-γR2/Hu-IFN-γR1 (γR2/γR1), Hu-IFN-γR2/Hu-IFN-γR1t_{456} (γR2/γR1t), Hu-IFN-γR2/Hu-IFN-αR1 (γR2/αR1), Hu-IFN-γR2/CRFB4 (γR2/CRF), Hu-IFN-γR2/IL-2Rγ_C (γR2/γ_C) to obtain stable transformants. In addition, 16-9 c lls were cotransfect d with an xpression vector encoding Hu-IFN-γR2/IL-2Rγ_C and an expression vector encoding murine Jak3 (γR2/γ_C + Jak3). Th se stable transformants xhibited a significant respons to Hu-IFN-γ (Figures 4B, C, D, E,

G, H, I). For all responsive cell lines the histograms represent th data for clonal cell populations. The Hu-IFN-γ did not induce MHC class I antig ns in 16-9 cells stably transfected with the expression vector encoding the Hu-IFN-γR2/Hu-IFN-αR2 (γR2/αR2) chimera (Figure 4F). As a control, it was shown that all cells responded to Hu-IFN-αA/D demonstrating that the MHC class I antigen could be induced in all cell lines (data not shown).

The Recruitment of Different Jaks into the IFN-γ Receptor Complex. It was reported that different receptors are associated with different Jak family members. Particularly, Hu-IFN-αR1 associates with Tyk2 [Barbieri *et al., supra*; Colamonici *et al., Mol. Cell. Biol.*, **14**:8133-8142 (1994)], Hu-IFN-γR2 associates with Jak2 [Kotenko *et al., supra*] and IL-2Rγ$_c$ associates with Jak3 [Russell *et al., supra*; Miyazaki *et al., supra*; Tanaka *et al., supra*]. Jak1 was shown to associate Hu-IFN-γR1 [Igarashi *et al., J. Biol. Chem.*, **269**:14333-14336 (1994); Sakatsume *et al., J. Biol. Chem.*, **270**:17528-17534 (1995)] and reported to associate with Hu-IFN-αR2 [Novick *et al., supra*]. In addition, Hu-IFN-γR1 probably weakly associates with Jak2 after oligomerization upon ligand binding [Kotenko *et al., supra*]. The ability of the intracellular domains of different receptors fused to the Hu-IFN-γR2 extracellular domain to recruit different members of the Jak family into the Hu-IFN-γ receptor complex and to determine if the recruited Jaks can be activated upon Hu-IFN-γ treatment was tested. First, the phosphorylation of Jak2 upon Hu-IFN-γ treatment in the cell lines expressing various chimeric receptors (Figure 5A) was determined. The phosphorylation of Jak2 in response to Hu-IFN-γ in cells was examined by immunoprecipitation with specific anti-Jak2 antibodies, followed by a western blot visualized with anti-phosphotyrosine antibodies. The phosphorylation of Jak2 only in γR2 cells (Figure 5A) was detected.

Inability to detect Jak2 activation in 16-9 cell lines responsive to Hu-IFN-γ containing the chimeric receptors γR2/αR1 and γR2/CRF suggested involvement of other Jaks in these cells. Since the Hu-IFN-αR1 associat s with Tyk2 [Barbieri *et al., supra*; Colamonici *et al., supra*], Tyk2 activation in cells expressing the chimeric γR2/αR1 chain was evaluated. With anti-Tyk2 antibodies which were develop d against human Tyk2, weak phosphorylation of

a protein of ~130 kD was detected which was precipitated with anti-Tyk2 antibodies in the cells expressing γR2/αR1 and γR2/CRF (Figure 5B). When the same blot was reprobed with anti-Tyk2 antibodies, these phosphorylated proteins corresponded to a band recognizable by anti-Tyk2 antibodies and likely represented hamster Tyk2 (Figure 5B, lower panels), but these proteins migrated slightly slower than human Tyk2 from lysates of COS-1 cells transiently transfected with a plasmid encoding human Tyk2 (data not shown). We observed the phosphorylated proteins of the same size in γR2/αR1 and γR2/CRF cells when immunoprecipitation was performed with anti-phosphotyrosine antibodies and the blot was probed with anti-Tyk2 antibodies (Figure 5C). The same differences in mobility of hamster Tyk2 from human Tyk2 from control lysates of COS-1 cells transiently transfected with a plasmid encoding human Tyk2 were observed (Figure 5C).

To confirm that the phosphorylated protein in γR2/αR1 and γR2/CRF cells was activated Tyk2, we stably cotransfected a plasmid encoding γR2/αR1 or γR2/CRF with a plasmid encoding human Tyk2 into the 16-9 cells. The new cell lines were designated γR2/αR1 + Tyk2 and γR2/CRF + Tyk2, respectively. As controls we also cotransfected a plasmid encoding either γR2, γR2/γR1 or γR2/αR2 with a plasmid encoding human Tyk2 into the 16-9 cells. The resultant cell lines were designated γR2 + Tyk2, γR2/γR1 + Tyk2 and γR2/αR2 + Tyk2, respectively. First, we performed immunoprecipitation with anti-Tyk2 antibodies from cellular lysates prepared from γR2/αR1 + Tyk2 and γR2/αR1 cells to evaluate the expression of exogenous human Tyk2 and endogenous hamster Tyk2. After blotting with anti-Tyk2 antibodies it was observed that human Tyk2 was expressed at a much higher level or was able to be precipitated with the antibodies to a greater extent than hamster Tyk2. We also observed the same differences in mobility of human Tyk2 expressed in the 16-9 cells and hamster Tyk2 as we observed above with Tyk2 expressed in COS-1 cells (Figure 5D). With these cell lines, we observed weak phosphorylation of Tyk2 in untreated γR2/αR1 + Tyk2 and γR2/CRF + Tyk2 cells as well as in untreated and treated γR2 + Tyk2, γR2/γR1 + Tyk2 and γR2/αR2 + Tyk2 cells. This is in agreement with the observation that overexpression of members of Jak family causes a low spontaneous level of phosphorylation of kinases in the absence of

ligand [Watling *et al., supra*; Müller *et al., Nature*, **366**:129-135 (1993); Silvennoinen *et al., supra*]. However, only in γR2/αR1 + Tyk2 and γR2/CRF + Tyk2 cells did we observe an enhancement of Tyk2 phosphorylation after Hu-IFN-γ treatment (Figure 5E). Thus, we showed that the intracellular domains of Hu-IFN-αR1 and Hu-CRFB4 linked to the extracellular domain of γR2 causes activation of Tyk2 instead of Jak2 which is regularly observed during IFN-γ signalling.

The chimeric receptor γR2/γ_c was able to render 16-9 cells responsive to human IFN-γ to a small extent as measured by class I MHC antigen induction (Figure 4H). It was shown that the IL-2Rγ$_c$ chain associates with Jak3 [Russell *et al., supra*; Miyazaki *et al., supra*; Tanaka *et al., supra*]. Thus, we examined whether Jak3 participates in the IFN-γ receptor complex in γR2/γ_c cells. We hypothesized that failure of Hu-IFN-γ to induce strong MHC class I antigen induction is due to low level of endogenous Jak3 expression in the 16-9 hamster ovary cells since Jak3 is normally only expressed in hemopoietic cells. To test the hypothesis that the low level of Jak3 limited the IFN-γ signalling in γR2/γ_c cells, we stably cotransfected a plasmid encoding γR2/γ_c with a plasmid encoding either Jak1, Jak2, Jak3, or Tyk2 into the 16-9 cells. Cell lines were designated γR2/γ_c + Jak1, γR2/γ_c + Jak2, γR2/γ_c + Jak3 and γR2/γ_c + Tyk2, respectively. Upon Hu-IFN-γ treatment, only γR2/γ_c + Jak3 cells showed strong enhancement in MHC class I antigen induction upon Hu-IFN-γ treatment (Figure 4I). All other cell lines showed the same level of responsiveness as γR2/γ_c cells (Figure4H). We confirmed the participation of Jak3 in IFN-γ signalling in the γR2/γ_c + Jak3 cells by immunoprecipitation experiments. Only in γR2/γ_c + Jak3 cells did we observe enhancement in Jak3 phosphorylation after Hu-IFN-γ treatment. After longer exposure, a low spontaneous level of Jak3 phosphorylation was observed in untreated γR2/γ_c + Jak3 cells and in treated and untreated γR2 + Jak3 cells used as a control similar to Tyk2 phosphorylation observed in cells overexpressing Tyk2 (see above). Thus, we showed that the γR2/γ_c chimeric receptor recruits Jak3 into the IFN-γ rec ptor complex due to association of the IL-2Rγ$_c$ intracellular domain with Jak3, and that IFN-γ induces phosphorylation of Jak3 in γR2/γ_c + Jak3 cells inst ad of phosphorylation of

Jak2. Therefore, the substitution of th intracellular domain of γ_c for γR2 accordingly substitut s Jak3 for Jak2 in th functional IFN-γ receptor compl x.

Jak1 was shown to be activated upon IFN-γ treatment by the in vitro kinase activation assay only in cell lines positive in MHC class I antigen induction.

IFN-γ Activates Stat1α through Jak Family Members other than Jak2. It was proposed that Jaks can contribute to the specificity of signal transduction by different IFNs, particularly, Tyk2 (which is active only during Type I IFN signalling) and Jak2 (which is active only during IFN-γ signalling) [Ihle *et al.*, *supra*]. To evaluate this hypothesis, we performed electrophoretic mobility shift assays with cell lysates prepared from the various transformants before and after treatment with Hu-IFN-γ (Figure 6). Since Stat1α homodimer formation occurs during IFN-γ signalling and Stat1α homodimers bind the GAS element with high specificity, we used oligonucleotides corresponding to the GAS element in the promoter region of the human IRF-1 gene as the phosphorylated probe [Yuan *et al.*, *Mol. Cell. Biol.*, **14**:1657-1668 (1994)]. The formation of Stat1α DNA binding complexes (Figure 6) was observed in all cell lines responsive to Hu-IFN-γ as determined by induction of MHC class I antigen (Figure 4). The Hu-IFN-γ induced activation of Stat1α in γR2/γ_c + Jak3 cells was increased to the level comparable to the level of Stat1α activation in all other Hu-IFN-γ responsive cell lines (data not shown). Because it was shown that other Stats can bind the same GAS element with different affinity [Seidel *et al.*, *Proc. Natl. Acad. Sci. USA*, **92**:3041-3045 (1995)], supershift assays were performed with specific anti-Stat1α antibodies to determine whether the GAS binding complexes observed in the tested cells are formed by Stat1α homodimers. The GAS binding complexes in all tested cells were shifted, indicating that Stat1α was activated in all Hu-IFN-γ responsive cells (Figure 6).

Discussion

Type I interferons (IFN-α and IFN-β) activate Jak1, Tyk2, Stat1 and Stat2 during signal transduction; Type II interf ron (IFN-γ) uses Jak1, Jak2 and Stat1 for signalling [V lazqu z *et al.*, *supra*; Watling *et al.*, *supra*; Müller *et al.*, *Nature*, **366**:129-135 (1993) and *EMBO*, **12**:4221-4228 (1993); Leung *et al.*, *supra*]. It

was proposed that th utilization of different kinases by a particular receptor complex could contribute to the specificity of signalling by different IFNs [Ihle *et al., supra*]. To evaluate this hypothesis, a series of experiments with chimeric receptors was undertaken.

The IFN-γ receptor complex whose components and signal partners are now well defined was employed as a model. It has been shown that the active receptor complex consists of two chains of IFN-γR1 (γR1) and likely two chains of IFN-γR2 (γR2) oligomerized upon primary binding of the IFN-γ homodimer to the IFN-γR1 [Greenlund *et al., J. Biol. Chem.*, **268**:18103-18110 (1993); Langer *et al., Proc. Natl. Acad. Sci. USA*, **91**:5818-5822 (1994); Marsters *et al., Proc. Natl. Acad. Sci. USA*, **92**:5401-5405 (1995); Walter *et al., Nature*, **376**:230-235 (1995); Kotenko *et al., supra*]. IFN-γR1 primarily associates with Jak1 [Igarashi *et al., supra*; Sakatsume *et al., supra*] and, perhaps, weakly with Jak2 after IFN-γR1 homodimerization [Kotenko *et al., supra*]. The intracellular domain of IFN-γR2 associates with Jak2 and brings Jak2 into the complex upon ligand binding [Kotenko *et al., supra*; Sakatsume *et al., supra*]. The expression of a kinase negative Jak1 mutant in a Jak1 negative (U4A) cell line can sustain an IFN-γ response, indicating that Jak1 predominantly plays a structural role in the normal functional IFN-γ receptor complex rather than catalytic role [Briscoe *et al., J. Interferon and Cytokine Res.*, **15**:S56 (1995)]. Thus PTK activity of Jak1 is dispensable for IFN-γ signalling. In contrast, the expression of a kinase negative Jak2 mutant in a Jak 2 negative (γ2A) cell line cannot sustain an IFN-γ response [Briscoe *et al., supra*], indicating that the PTK activity of Jak2 is absolutely necessary for IFN-γ signalling. Thus, we tested whether substitution of Jak2 by other kinases would change the specificity of signal transduction by IFN-γ. For this purpose the intracellular domains of other receptor chains was substituted for the intracellular domain of Hu-IFN-γR2 to recruit kinases other than Jak2 into the IFN-γ receptor complex. The extracellular domain of Hu-IFN-γR2 was fused to the transmembrane and intracellular domains of either the Hu-IFN-γR1 (γR2/γR1), Hu-IFN-αR1 (γR2/αR1), Hu-IFN-αR2 (γR2/αR2), Hu-CRFB4 (γR2/CRF) or Hu-IL-2Rγ_c chain (γR2/γ_c) (Figure 1). The chimeric chains w re expressed in 16-9 c lls, Chinese hamst r ovary cells expressing Hu-IFN-γR1. By crosslinking [Kotenko *et al., supra*] it was shown that all chimeric receptors were expressed

on th c ll surfac and wer able to participate in formation of th xtracellular IFN-γ receptor complex (Figure 3). We then d termined biological responsiveness of the cells to Hu-IFN-γ as measured by MHC class I antigen induction (Figure 4). The chimeric receptors γR2/γR1, γR2/αR1, γR2/CRF and γR2/γ_c rendered the 16-9 cells responsive to Hu-IFN-γ (Figure 4C, E, G, H). In contrast, the chimeric receptor γR2/αR2 did not support signal transduction by Hu-IFN-γ (Figure 4F). Finally, we investigated the activation of Jaks and Stats to determine whether the specificity of signal transduction is altered in cells expressing these chimeric receptors.

In γR2/αR1 cells detected phosphorylation of Tyk2, but not Jak2 (Figure 5A, B, C and E) was detected. This is in agreement with observation that the IFN-αR1 (αR1) intracellular domain associates with Tyk2 [Barbieri *et al.*, *supra*; Colamonici *et al.*, *supra*]. Similarly, it was shown that Hu-IFN-γ induces phosphorylation of Tyk2, but not Jak2 in γR2/CRF cells (Figure 5A, B, C and E). Thus, the intracellular domain of CRFB4 or Hu-IFN-αR1 fused to the extracellular domain of Hu-IFN-γR2 recruits Tyk2 into the IFN-γ receptor complex and allows Hu-IFN-γ to signal through activation of Tyk2 instead of Jak2, as usually occurs during IFN-γ signalling. Thus, the activation of Jak2 *per se* is not necessary for signal transduction by IFN-γ and Tyk2 can substitute for Jak2.

The γR2/γR1 chimeric chain brings an additional molecule of Jak1 into the IFN-γ receptor complex. Although phosphorylation of Jak2 in γR2/γR1 cells was not detectable (Figure 5A), there may be some involvement of Jak2 in signal transduction by IFN-γ in γR2/γR1 cells, arising through a weak interaction between dimerized IFN-γR1 and Jak2 [Kotenko *et al.*, *supra*]. There are two regions of the IFN-γR1 intracellular domain important for signalling, the membrane proximal region, with a possible Jak1 association site [Farrar *et al.*, *J. Biol. Chem.*, **266**:19626-19635 (1991)], and the membrane-distal region around Tyr457 [Cook *et al.*, *supra*; Farrar *et al.*, *Proc. Natl. Acad. Sci. USA*, **89**:11706-11710 (1992)], the Stat1α recruitment site [Greenlund *et al.*, *supra*]. The presence of the Stat1α recruitment site on this chimeric receptor chain is not required for activity becaus γR2/γR1t_{456} cells that also express the normal Hu-IFN-γR1 chain are still responsive to Hu-IFN-γ (Figur 4D).

To investigat further th ability of Jaks to substitute for each other γR2/γ_c cells were examined. The IL-2Rγ_c chain is a common chain for a broad range of receptor complexes for such cytokines as IL-2, IL-4, IL-7, IL-9 [for review see Kishimoto *et al.*, *Cell*, **76**:253-262 (1994)] and IL-15 [Giri *et al.*, *EMBO*, **13**:2822-2830 (1994)]. The intracellular domain of the IL-2Rγ_c chain associates with Jak3 [Russell *et al.*, *supra*; Miyazaki *et al.*, *supra*; Tanaka *et al.*, *supra*]. The expression of both the IL-2Rγ_c chain and Jak3 are restricted to certain cell types [Minami *et al.*, *Ann. Rev. Immunol.*, **11**:245-267 (1993); Johnston *et al.*, *supra*; Witthuhn *et al.*, *supra*]. MHC class I antigen induction in γR2/γ_c cells was observed in response to Hu-IFN-γ (Figure 4H), but the induction was weaker than in all other cell lines expressing chimeric receptors (Figure 4B, C, E, G). However, by co-expression of γR2/γ_c together with either Jak1, Jak2, Jak3 or Tyk2, only Jak3 was shown to be able to restore the biological responsiveness of γR2/γ_c cells to Hu-IFN-γ (Figure 4H, I). We further showed that Jak3 is phosphorylated upon Hu-IFN-γ treatment of these cells (Figure 5F). Thus, Jak3 can functionally substitute for Jak2 in the active IFN-γ receptor complex.

Since the formation of the Stat1α homodimeric DNA-binding complex as the only binding complex is specific for IFN-γ signalling, we investigated whether substitution of other kinases for Jak2 would change the specificity of the Jak-Stat signal transduction pathway. The formation of Stat1α DNA binding complexes was observed in all cell lines positive in MHC class I antigen induction (Figure 6). By the supershift assay with specific anti-Stat1α antibodies we further showed that the GAS binding complexes consisted of Stat1α homodimers (Figure 6). Thus, we concluded that other members of the Jak family recruited to the IFN-γ receptor complex can substitute for Jak2 without changing the specificity of IFN-γ signalling in the Jak-Stat pathway. That is, the activation of Stat1α during IFN-γ signalling does not require the specific participation of Jak2.

It was shown r cently that th re are two splice variants of the IFNAR2 gene: a short form, Hu-IFN-αR2b (αR2b) and a long form, Hu-IFN-αR2c (αR2c). Th y share the same xtrac llular and transmembran domains, but the stop c don of the αR2b is spliced out in the αR2c cDNA r sulting in a longer intracellular

domain f r αR2c. Only the αR2c variant can complement the mutation in U5A cells defective in IFN-α signalling and sustain the IFN-α respons [Lutfalla *et al.*, *supra*]; and together with the Hu-IFN-αR1 chain can reconstitute an active human IFN-α receptor complex in mouse cells [Colamonici *et al.*, *supra*]. Since only the αR2b variant was known at the time of this work, the αR2b intracellular domain was used. After becoming aware of the existence of the αR2c chain, a chimera γR2/αR2c was constructed and expressed in 16-9 cells. Hu-IFN-γ induced MHC class I antigens in γR2/αR2c cells to the same extent as in γR2 cells. Thus, because we showed that the γR2/αR2b (γR2/αR2) chimera was unable to support IFN-γ signalling (Figure 4F), we conclude that the intracellular domain of the short form of αR2, αR2b, is unable to bring PTK activity to the IFN-γ receptor complex in contrast to the αR2c intracellular domain. The box 1 motif (proline rich sequence) which is required for association with Jaks [Lebrun *et al.*, *J. Biol. Chem.*, **270**:10664-10670 (1995); Tanner *et al.*, *J. Biol. Chem.*, **270**:6523-6530 (1995)] is not present in the short αR2b chain [Colamonici *et al.*, *supra*]. This can explain the failure of αR2b to complement U5A cells (cells lacking αR2b and c) for IFN-α signalling [Lutfalla *et al.*, *supra*], as well as the failure to render mouse cells responsive to Hu-IFN-α [Colamonici *et al.*, *supra*].

In addition to demonstrating that other Jaks can substitute for Jak2 in IFN-γ signal transduction, we provide here evidence that the intracellular domain of CRFB4, a class II cytokine receptor with unknown function [Lutfalla *et al.*, *supra* (1993)] associates with Tyk2. Therefore, CRFB4 is likely a component of a ligand-receptor system which activates Tyk2 during signal transduction. Tyk2 was shown to be activated by IFN-α, CNTF-related cytokines, IL-10 and IL-12 [Velazquez *et al.*, *supra*; Barbieri *et al.*, *supra*; Lütticken *et al.*, *supra*; Stahl *et al.*, *Science*, **263**:92-95 (1994); Finbloom and Winestock, *supra;* Bacon *et al.*, *supra*]. Since the components of a given cytokine-receptor complex belong to the same class of the cytokine receptor superfamily, it is most likely that CRFB4 is involved in IFN-α or IL-10 receptor complexes, as all cloned subunits of these rec ptors ar members of the sam class as CRFB4 [Uzé *et al.*, *supra*; Liu *et al.*, *J. Immunol.*, **152**:1821-1829 (1994); Novick *et al.*, *supra*] unlike the components of th CNTF-related cytokine receptor subunits and the IL-12 receptor. Thus, th use of chimeric receptors provides a method to study the

properties of intracellular domains of receptors from unknown or incompletely characterized ligand-receptor complexes as illustrated with the chimeric receptors containing the intracellular domains of Hu-IFN-αR1, Hu-IFN-αR2 or CRFB4.

Of most importance, the results described herein show that the Jaks are interchangeable for the Jak-Stat signal transduction pathway. The results allow us to expand our model for IFN-γ signalling [Kotenko *et al.*, *supra*], which may be general for class II cytokine receptors (Figure 7). The signal transducing receptor chains can be divided into two classes: (1) the actual Signal Transducers (ST), containing Stat (or other SH2 domain containing protein) Recruitment Sites (SRS) and Jak Association Sites (JAS); and (2) Helper Receptors (HR), containing only JAS, but no SRS. The primary function of the HR is to bring additional PTK activity to the receptor complex upon ligand binding. They do not contain functionally important Tyr residues. Thus far two receptors fit this HR category: the IL-2Rγ$_c$ and IFN-γR2 chains, as it was shown that the substitution of Tyr residues within their intracellular domains does not change the ability of these receptors to support signal transduction [Lai *et al.*, *FASEB J.*, **9**:A1021 (5917) (1995); Bach *et al.*, *FASEB J.* **9**:A1021 (5919) (1995)]. The CRFB4 chain and probably IFN-αR1 are other candidates for helper receptors. In those cases where homodimerization of a single receptor chain appears sufficient for signal transduction and its intracellular domain contains all the JAS and SRS regions necessary and sufficient for signal transduction (as in the case of EPO-R, GHR or ProR), the activation of a single Jak2 is observed and a separate HR chain is not required [Argetsinger *et al.*, *Cell*, **74**:237-244 (1993); Witthuhn *et al.*, *Nature*, **370**:153-157 (1993); Campbell *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**:5232-5236 (1994); DaSilva *et al.*, *J. Biol. Chem.*, **269**:267-270 (1994); David *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**:7174-7178 (1994); Dusanter-Fourt *et al.*, *EMBO J.*, **13**:2583-2591 (1994); Rui *et al.*, *J. Biol. Chem.*, **269**:5364-5368 (1994)]. We hypothesize that the intracellular domains of the HR can be associated with any Jak and do not provide any specificity for signal transduction. Only their extracellular domains are specific for particular ligand receptor complexes. The Jaks show preferential specificity for association with the receptor intracellular domains just like the Stats [Heim *et*

al., *supra*; Stahl *et al.*, *Science*, **267**:1349-1353 (1995)], but the kinase domains *per se* are promiscuous. Finally, it was hypothesized that the Jaks do not contribute to the specificity of signal transduction in the Jak-Stat pathway, inasmuch as they do not posses a preferential specificity for Stat activation.

EXAMPLE 2: MHC Class I Antigen Induction in Hamster Cells in Response to IL-10

The observation that the expression of Hu-IL-10R alone in mouse or COS7 cells [Liu *et al.*, *supra*] was sufficient to reconstitute some IL-10 signalling suggested that a second chain of the IL-10 receptor complex is expressed widely and is not species-specific between mouse, monkey, and human cells. In our experiments with COS1 cells transfected with the IL-10R chain, we observed little or no activation of Stat1 and Stat3. When both IL-10R and CRFB4 chains were transfected together into COS1 cells, there was a very nice activation of these Stats.

On the other hand, we did not see the activation of Stats in hamster cells stably transfected with IL-10R upon IL-10 treatment as measured by electrophoretic mobility shift assay. Since the activity of IL-10 is restricted to certain cell types [Ho and Moore, *Ther. Immunol.*, 1:173-85 (1994)] and since a ready method to determine the biological activities of IL-10 in hamster cells was not available, a chimeric receptor approach was adopted (see Example 1). The intracellular domain of the IL-10R was substituted with the intracellular domain of the IFN-γR1. To construct chimera IL-10R/γR1, the PCR reaction was performed with 5'-CGGGGTACCCAGGATGCTGCCGTGCC-3' (SEQ ID NO:25) and 5'-ATCGCTAGCCAGTTGGTCACGGTGAAATAC-3' (SEQ ID NO:13) primers. The PCR product was digested with *Nhe*I and *Kpn*I restriction endonucleases and ligated into the *Nhe*I and *Kpn*I sites of the plasmid pEF2-γR1. The plasmid was designated pEF2-IL-10R/γR1. It was shown that the IFN-γR1 intracellular domain is a signal transducing chain of the IFN-γR1 complex that recruits Stat1α to the complex (Example 1). To enable the IFN-γR1 chain to initiate signalling, the IFN-γR1 chain requires an additional tyrosine kinase activity which is brought

into the normal IFN-γ receptor complex on the intracellular domain of the IFN-γR2 (Example 2). Although this function is normally performed by Jak2 kinase associated with the IFN-γR2 intracellular domain, it can also be performed by any tyrosine kinase of the Jak family, including Tyk2, associated with the intracellular domain of the IFN-γR2/X chimeric receptors (Examples 1 and 2). Since Tyk2 is associated with the CRFB4 intracellular domain (Example 2), the IL-10R/γR1 chimeric receptor and CRFB4 chains were postulated to interact upon IL-10 binding so that the intracellular domains of these two chains would mimic the interaction that occurs between the intracellular domains of the normal IFN-γ receptor complex, and would thus initiate signal transduction. Because IFN-γ induces MHC class I antigen expression, we used this cell surface marker was used to determine if IL-10 would signal through the IL-10R/γR1 chain expressed together with the CRFB4 receptor chain.

When the IL-10R/γR1 chimera was expressed alone, it was not able to support signalling upon IL-10 treatment as measured by MHC class I antigen induction (Figure 9). However, hamster cells stably transfected with both IL-10R/γR1 and CRFB4 chains showed the MHC class I antigen induction in response to IL-10 (Figure 9). This supports the previous observation that the CRFB4 chain is the second subunit of the IL-10 receptor complex. The CRFB4 chain brings the associated Tyk2 to the IL-10 receptor complex upon IL-10 binding and thus initiates signal transduction by IL-10.

EXAMPLE 3: Chimeric Erythropoietin Interferon Gamma Receptors

The present Example demonstrates preparation of a functional chimeric EpR/γR1 and EpR/γR2 receptors. The role of IFN-γR2 in the IFN-γ receptor signal transduction complex and the requirements of multimer formation between the two chains was evaluated. A series of chimeric receptors between EpoR and IFN-γR1 and IFN-γR2 were constructed. The results suggest that the essential and perhaps only function of the IFN-γR2 intracellular domain is to bring Jak2 into the signal transduction complex. Furthermore, IFN-γR2 serves an important structural role in the configuration of the IFN-γ receptor signal transduction complex.

As discussed above, signal transduction by interferon gamma (IFN-γ) involves oligomerization of the two interferon gamma receptor (IFN-γR) subunits, receptor chain 1 (IFN-γR1) which is the ligand-binding chain and the second chain of the receptor (IFN-γR2), upon binding of the ligand. IFN-γ is a symmetrical homodimer that binds two IFN-γR1 chains in a species-specific manner, followed by a species-specific interaction between the extracellular domains of IFN-γR1 and IFN-γR2. Two kinases, Jak1 and Jak2 are involved in IFN-γR signalling. The IFN-γR1 chain binds Jak1 and the IFN-γR2 chain interacts with Jak2.

The erythropoietin receptor (EpoR), like the IFN-γR, lacks intrinsic kinase activity. It interacts with Jak2 which is activated upon ligand-induced EpoR dimerization. The erythropoietin receptor consists of a single chain which possesses a Jak-association site and a Stat-recruitment site, and appears to be sufficient for ligand-binding as well as signal transduction. On the contrary, the IFN-γR1 chain, which also possesses both a Jak-association site and a Stat-recruitment site is alone unable to transduce a signal when bound to its ligand.

Materials And Methods

Reagents, Restriction Endonucleases and Other Enzymes. Erythropoietin was produced recombinantly. Restriction endonucleases were from Boehringer Mannheim Biochemicals and New England Biolabs, T4 DNA ligase was from United States Biochemicals; [α-^{32}P]dCTP was from New England Nuclear. All other reagents were of analytical grade and purchased from Sigma.

Cells and Media. CHO-B7 cells represent the Chinese hamster ovary cell line (CHO-K1) containing a transfected human HLA-B7 gene [Jung *et al.*, 1990, *supra*]. The 16-9 hamster x human somatic hybrid cell line is a CHO-K1 derivative containing a translocation of the long arm of human Chromosome 6 and the human HLA-B7 gene [Soh *et al.*, 1993, *supra*]. These cells were maintained in F-12 medium (HAM, Life Technologies) containing 10% heat-inactivated fetal bovine serum (Sigma). Transfections were carried out with the DOTAP

transfection reagent (Boehringer Mannheim Biochemicals) according to the manufacturer's protocol and the transfected cells were maintained in F12 medium containing 450 μg/ml geneticin (antibiotic G418).

Construction of Chimeric Receptors. The EpoR expression plasmid was made by cloning the *Eco*RI-*Afl*III fragment of the human EpoR cDNA p18R [Jones *et al.*, *Blood* **76**:31-35 (1990)] into the *Eco*RI and *Eco*RV sites of the eukaryotic expression vector pcDNA3 (Invitrogen). The construction of plasmids expressing Hu-IFN-γR1 and Hu-IFN-γR2 chains from cDNA under the control of CMV promoter has been previously described [Kumar *et al.*, 1989, *supra*; Jung *et al.*, 1990, *supra*; Kotenko *et al.*, 1995, *supra*, 1996]. For ease of construction of the various chimeric receptors, the polymerase chain reaction (PCR) was employed to incorporate a unique *Nhe*I site at the 3′ end of the extracellular domain (EC) and at the 5′ end of the transmembrane-intracellular domains (IC) of the receptors. The primers were designed to code for the three amino acids Trp, Leu and Ala, which are commonly found in the transmembrane domain of several proteins, encompassing the *Nhe*I site. The extracellular portions of EpoR, Hu-IFN-γR1 and Hu-IFN-γR2, containing an *Nhe*I site (designated $EpoR_{EC}$/*Nhe*I, $\gamma R1_{EC}$/*Nhe*I and $\gamma R2_{EC}$/*Nhe*I) were generated by PCR from the respective cDNAs as templates with the use of the T7 primer (5′-TAATACGACTCACTATA-3′) (SEQ ID NO:26) and the internal primers 5′-GCCGCTAGCCAGGGGTCCAGGTCGCTAGGCG-3′, (SEQ ID NO:27) corresponding to nucleotides 1874-1893 of p18R [Jones *et al.*, 1990, *supra*], 5′-GTGGCTAGCCAAGAACCTTTTATACTGCT-3′ (SEQ ID NO:28) corresponding to nucleotides 779-785 of Hu-IFN-γR1 [Aguet *et al.*, 1988, *supra*] and 5′-ATCGCTAGCCATTGCTGAAGCTCAGTGGAGG-3′ (SEQ ID NO:13) corresponding to nucleotides 1370-1390 of Hu-IFN-γR2 [Soh *et al.*, 1994, *supra*]. The intracellular portions of the various receptors with the unique *Nhe*I site at the 5′ end of the transmembrane domain (designated $EpoR_{IC}$/*Nhe*I, $\gamma R1_{IC}$/*Nhe*I and $\gamma R2_{IC}$/*Nhe*I were generated by PCR on corresponding cDNA templates with the use of the SP6 primer (5′-ATTTAGGTGACACTATA-3′) (SEQ ID NO:29) and the internal primers

5'-GTGGCTAGCGACGCTCTCCCTCATCCTCG-3' (SEQ ID NO:30) corresponding to nucleotides 1902-1921 of p18R, 5'-GTGGCTAGCGATTCCAGTTGTTGCTGCTTTAC-3' (SEQ ID NO:31) corresponding to nucleotides 792-814 of Hu-IFN-γR1 and 5'-GTGGCTAGCGATCTCCGTGGGAACATTT-3' (SEQ ID NO:32) corresponding to nucleotides 1398-1416 of Hu-IFN-γR2. The *Nhe*I site in each primer is underlined. The PCR products encoding the extracellular domains were incubated with T4 DNA polymerase and dNTPs to generate blunt ends and subsequently digested with the restriction endonucleases *Eco*RI($EpoR_{EC}$/*Nhe*I and $\gamma R2_{EC}$/*Nhe*I) or *Bam*HI ($\gamma R1_{EC}$/*Nhe*I), and cloned into the *Eco*RV and *Eco*RI/*Bam*HI sites of the expression vector pcDNA3 (Invitrogen) to yield the plasmids $pEpoR_{EC}$, $p\gamma R1_{EC}$ and $p\gamma R2_{EC}$. The PCR products encoding the intracellular domains of the various receptors were treated with T4 DNA polymerase to generate blunt ends, digested with *Xba*I restriction endonuclease and cloned into the *Eco*RV and *Xba*I sites of pcDNA3 to yield the plasmids $pEpoR_{IC}$, $p\gamma R1_{IC}$ and $p\gamma R2_{IC}$. To introduce the Stat1α binding site of Hu-IFN-γR1 into the cytoplasmic domain of EpoR, two-step asymmetric PCR was carried out sequentially on Hu-IFN-γR1 cDNA and $pEpoR_{IC}$ cDNA templates with vector primers and the internal primer CTTGTCCTTCTGTTTTTATTTCagagcaagccacatagetggg (SEQ ID NO:33). The upper case letters denote sequences of Hu-IFN-γR1 and the lower case letters represent sequences of EpoR. The Hu-IFN-γR2 chain with the Stat1α binding site of Hu-IFN-γR1 was constructed by restriction enzyme digestion of $p\gamma R2_{IC}$ and IFN-γR1 cDNA with *Bsp*E1 and *Ava*I respectively, followed by ligation. For construction of the chimeric receptors, plasmids encoding the suitable extracellular or intracellular domains were digested with *Nhe*I and *Xba*I restriction endonucleases and ligated together. All constructs were sequenced for verification of the entire nucleotide sequence of the receptor. Sequencing was done in an ABI 373 automated DNA sequencer with dideoxy dye-terminator chemistry.

Electrophoretic Mobility Shift Assays (EMSA). EMSAs were performed with the 22 base pair sequence containing a Stat1α binding site (5'GATCGATTTCCCCGAAATCATG-3') (SEQ ID NO:24) corresponding to the GAS element in the promoter region of the human IRF-1 gene [Yuan *et al.*, *Mol. Cell. Biol.* **14**:1657-1668 (1994)]. Two oligonucleotides 5'-GATCGATTTCCCCGAAAT-3' (SEQ ID NO:34) and 5'-CATGATTTCGGGGAAATC-3' (SEQ ID NO:35) were annealed by incubation for 10 minutes at 65°C, 10 minutes at 37°C and 10 minutes at 22°C, and labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I by the filling-in reaction [Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual*, 2nd ed., Cold Spring Harbor Press (1989)]. Whole-cell extracts were prepared as follows [Dignam *et al.*, *Nucl. Acids Res.* **11**:1475-1489 (1983)]. Cells were grown to confluence in 6-well plates, and harvested by scraping in ice-cold PBS. Cells from each well were washed with 1.0 ml cold PBS, pelleted and resuspended in 100 μl of lysis buffer (10% glycerol, 50 mM Tris•HCl, pH 8.0, 0.5% NP-40, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, and 1 mM Na_3VO_4, 3 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin). After 30 minutes on ice, the extracts were centrifuged for 5 minutes at full speed in a microfuge and the supernatant was recovered for use in the assay and stored at -80°C.

EMSA reactions contained 2.5 μl of the whole cell extracts, 1 ng ^{32}P-labeled probe (specific activity 10^9 cpm/μg), 24 μg/ml BSA, 160 μg/ml poly(dI:dC), 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 4.0% Ficoll (Pharmacia), 40 mM KCl, 0.1 mM EGTA, and 0.5 mM DTT in a total volume of 12.5 μl. For the supershift assay 1 μl of a 1:10 dilution of anti-Stat1α antibody was included in the reaction. Competition experiments contained a 100-fold excess of the unlabelled oligonucleotide. Reactions were incubated at 24°C for 20 minutes. Then 8 μl of the reaction mixture was electrophoresed at 400V for 3-4 hours at 4°C on a 5% polyacrylamide (19:1 acrylamide:bisacrylamide) gel. The dried gel was exposed to Kodak XAR-5 film with an intensifying screen for 12 hours at -80°C.

Antibodies. Rabbit anti-Jak1, anti-Stat 1, and anti-Jak 2 antibodies are described in Example 1.

Immunoprecipitations and Blottings. Cells were stimulated with Hu-IFN-γ (1000 units/ml) or Epo (50 units/ml) for 10 minutes at 37°C. Immunoprecipitations and blottings were performed as described [Kotenko *et al.*, 1995, *supra*].

Cytofluorographic analysis. Cytofluorographic analysis of cells for surface expression of class I MHC antigens was performed as described previously [Cook *et al.*, *Proc. Natl. Acad. Sci. USA* **89**:11317-11321(1992); Soh *et al.*, 1993; *supra*] with mouse anti-human-HLA B-7 monoclonal antibody (W6/32) and FITC-conjugated goat anti-mouse IgG.

Results

Construction of Chimeric Receptors. The schematic illustration of the various chimeric receptor molecules that were produced is shown in Figure 10. In one set of chimeric constructs the extracellular domain of the EpoR was spliced to the transmembrane domain and the cytoplasmic domain of each of the two IFN-γR subunits. In the other set of chimeras the transmembrane and intracellular domain of EpoR was attached to the extracellular domain of IFN-γR1 and IFN-γR2.

Class I MHC Antigen Induction. In order to investigate the role of the intracellular domain of IFN-γR2 in the signal transduction complex of IFN-γ, a chimeric receptor chain consisting of the extracellular domain of IFN-γR2 and the intracellular domain of EpoR was constructed. This chimeric construct, γR2/EpoR, and the native IFN-γR2 subunit were separately transfected into CHO-B7 as well as 16-9 cells. The ability of the transfected chimeric cDNA to transduce a signal upon induction with Hu-IFN-γ was assayed by measurement of enhanced MHC class I antigen expression in the transfected cells. CHO-B7 cells transfected with IFN-γR2 or γR2/EpoR cDNA showed no response to Hu-IFN-γ as they lack the ligand-binding receptor subunit, Hu-IFN-γR1 (data not shown).

CHO-16-9 cells, which contain human Chromosome 6q and express the Hu-IFN-γR1 subunit, showed no response to Hu-IFN-γ (Figure 11, panel A) but when stably transfected with expression vectors encoding Hu-IFN-γR2 cDNA or γR2/EpoR chimera, exhibited enhanced cell surface expression of class I MHC antigens in response to Hu-IFN-γ (Figure 11, panels B and C). To assess how effectively the intracellular domain of EpoR could substitute for the intracellular domain of the IFN-γR2 subunit, the induction of MHC class I antigens as a function of the concentration of IFN-γ used to activate the stably transfected cells was measured. As depicted graphically in Figure 12, the chimeric γR2/EpoR subunit was approximately as effective as native IFN-γR2 in stimulating MHC class I antigen induction in response to Hu-IFN-γ. However, there appeared to be a slightly lower induction of MHC class I antigens in the cells containing the chimeric γR2/EpoR than in the cells containing the native Hu-IFN-γR2 chain at each concentration of Hu-IFN-γ used (Figure 12).

The fact that the EpoR intracellular domain can be substituted for the Hu-IFN-γR2 intracellular domain shows that another receptor domain that can recruit Jak2 into the signal transduction complex can substitute for the intracellular domain of Hu-IFN-γR2. It is possible that the intracellular domain of Hu-IFN-γR2 has additional functions that are also supported by the intracellular domain of EpoR. Since Jak2 is absolutely essential for signal transduction of IFN-γ [Witthuhn *et al.*, 1993, *supra*; Müller *et al.*, *Nature* **366**:129-135 (1993)], it is clear that the intracellular domain of EpoR carries out this function effectively.

Various chimeric receptors between the EpoR and Hu-IFN-γR1 and Hu-IFN-γR2 subunits were constructed in order to gain an understanding of the events involved in the oligomerization of the IFN-γ receptor subunits leading to signal transduction in response to IFN-γ. CHO-16-9 cells were stably transfected with expression vectors coding for EpoR, EpoR/γR1, EpoR/γR2, the combination of EpoR/γR1 and EpoR/γR2, and γR1/EpoR. In response to Epo, the EpoR transfectants showed no response (Figure 11, panel D). The EpoR/γR1 transfectants showed

some enhancement of expression of MHC class I antigens (Figure 11, panel E). However, the transfectants containing both EpoR/γR1 and EpoR/γR2 chains exhibited greater expression of MHC class I antigens (Figure 11, panel F, Figure 13). This shows that the Hu-IFN-γR1 chain, by itself, possesses or associates with, all the requisite components for signal transduction. Its association with the Hu-IFN-γR2 chain upon ligand binding greatly enhances its biological activity, as indicated by the higher activity of the cell line transfected with the two chains: EpoR/γR1 and EpoR/γR2. The observation that, on activation by the ligand, the EpoR/γR1 chimeric receptor is able to transduce a signal (Figure 11, panel E) whereas the native IFN-γR1 receptor is not, suggested that the basic difference between the two receptor complexes lies in their physical structure, particularly a constraint in the interaction of the extracellular domains. The EpoR/γR1 chimeric receptor dimer most likely permits interaction of the two IFN-γR1 intracellular domains. In the native IFN-γR1 dimer however, the intracellular domains of the two chains are too far apart for them to interact with each other [Walter *et al.*, *Nature* **376**:230-235 (1995)]. Consistent with this idea is the observation that cells transfected with expression vector coding for EpoR(p91) chimeric cDNA (EpoR with the p91 recruitment site from IFN-γR1) responds to Epo with enhanced expression of class I MHC antigens while the γR1/EpoR(p91) transfectants were unresponsive (Figure 11, panels G and H). Furthermore, the γR1/γR2(p91) receptor chain is unable to transduce a signal upon binding ligand, whereas the cells expressing the EpoR/γR2(p91) chimeric receptor exhibited enhanced class I MHC antigen expression in response to activation by ligand (data not shown).

Activation of Stat Proteins. Since one of the earliest events in signal transduction by IFN-γ is activation of the latent transcription factor Stat1α, Stat activation in cells expressing wild-type and chimeric receptors in response to IFN-γ was analyzed. As shown in Figure 14, IFN-γ stimulation resulted in Stat1α activation in transfected cell lines expressing native IFN-γR2 or γR2/EpoR chains. Similarly Epo caused activation of Stat1α in transfected cell lines expressing EpoR/γR1 and both EpoR/γR1 and EpoR/γR2 receptor chains (Figure 15). Consistent with the

small enhancement in surface expression of class I MHC antigens in cells expressing EpoR/γR1 in response to Epo, Stat1α activation was also lower in these cells compared to cells expressing both EpoR/γR1 and EpoR/γR2 chains. Activation of p91 was also observed in cells expressing EpoR(p91) (Figure 15). Furthermore, cells expressing those chimeric receptors containing the EpoR intracellular domain exhibited activation of Stat5 in addition to Stat1α (Figure 14). Stat5 is phosphorylated on tyrosine in response to Epo in certain cell lines [Ihle and Kerr, *Trends in Genetics* 11:69-74 (1995)]. The Stat1α complex is supershifted specifically by the addition of anti-Stat1α antibody and similarly anti-Stat5 antibody causes a specific shift of the activated Stat5 complex. Addition of 100-fold molar excess of unlabeled GAS oligonucleotide eliminates both Stat1α and Stat5 activated complexes and inclusion of both anti-Stat1α and anti-Stat5 antibodies in the reaction resulted in a shift of both bands.

Activation of Jak Kinases. Jak kinases play a central role in mediating signal transduction by various cytokine receptors in response to binding of ligand. Ligand binding induces receptor aggregation and, in some instances, increases the affinity of the cytoplasmic domain of the receptor for Jak kinases. The resulting increase in Jak kinase concentration in the receptor complex permits their cross-phosphorylation and activation of kinase activity. IFN-γ activates Jak1 and Jak2 kinases [Müller *et al.*, 1993, *supra*] whereas Epo activates Jak2 [Witthuhn *et al.*, 1993, *supra*] during signal transduction. Thus, the ability of the various chimeric receptors to activate Jak1 and Jak2 kinases in response to binding of ligand was tested.

Phosphorylation of Jak1 and Jak2 (Figure 16) was examined by immunoprecipitation of cellular lysates with antiphosphotyrosine antibodies followed by a Western blot visualized with specific anti-Jak1 and anti-Jak2 antibodies. Both Jak1 and Jak2 were phosphorylated in response to Hu-IFN-γ treatment in 16-9 cells expressing parental IFN-γR2 or chimeric γR2/EpoR receptor. Induction with Epo phosphorylated both Jak1 and Jak2 kinase in the cell

line expressing both EpoR/γR1 and EpoR/γR2 chains. However, in the cell line expressing only the chimeric EpoR/γR1 receptor, only Jak1 kinase was phosphorylated in response to Epo, as the cytoplasmic domain of this chimeric receptor lacks a Jak2 association site. The cell line transfected with the γR1/EpoR chimeric receptor did not exhibit phosphorylation of either Jak1 or Jak2 kinase upon IFN-γ treatment. This observation is consistent with the notion that, in an IFN-γR1 dimer, the intracellular domains of the two chains are too far apart to permit crossphosphorylation of the associated Jak kinases.

Discussion

For many hormones and cytokines, the conversion of the extracellular ligand-binding event to the intracellular signal involves a change in the oligomeric structure of the receptor. This observation has led to the notion that changes in receptor oligomerization may initiate signal transduction. Depending on the ligand, this can take the form of receptor homodimers (Epo, GH), heterodimers (CNTF, LIF), homotrimers (TNF), and more complex assemblies [reviewed by Stahl and Yancopoulos, *Cell*, 74:587-590 (1993)]. There is no clear example yet of a protein hormone, growth factor, or cytokine receptor having just a single functional transmembrane chain without a requirement for dimerization or oligomerization.

In the case of IFN-γ, its binding to the IFN-γR1 receptor chain ensures the specificity of the signal. The subsequent oligomerization, involving IFN-γR2, initiates the signal transduction events. After binding IFN-γ, when the receptor dimerizes, the IFN-γR1 subunits are phosphorylated on Tyr-457 [Greenlund *et al.*, 1994, *supra*; Kotenko *et al.*, 1995, *supra*] by activation of Jak1 and Jak2 kinases, followed by activation of the transcription factor Stat1α [Shuai *et al.*, 1993, *supra*]. A major function of receptor dimerization may be to bring two receptor-associated kinases together for transactivation. Earlier studies have shown the requirement for the formation of a heteromeric ternary complex comprised of two IFN-γR1 chains and two IFN-γR2 chains together with the IFN-γ ligand as the

signal-transducing complex [Kotenko *et al.*, 1995, *supra*]. The cytoplasmic domain of the IFN-γR2 subunit binds Jak2 and, upon binding of the ligand and association with the IFN-γR1 chain, serves to bring Jak2 kinase into the signal transduction complex [Kotenko *et al.*, 1995, *supra*]. This is a crucial event since deletion of the membrane proximal region of the intracellular domain of the IFN-γR2 chain, which encompasses the Jak2 association site, completely abrogates its ability to transduce signals in response to IFN-γ. Thus we propose that the major function of the IFN-γR2 subunit is to bring a Jak2 kinase in apposition to the Jak1 kinase on the IFN-γR1 subunit. This juxtaposition of the two Jak kinases permits their effective reciprocal transphosphorylation. This is supported by the observation that the IFN-γR2/EpoR chimeric receptor appears to be as effective as the native IFN-γR2 receptor chain in supporting signal transduction in response IFN-γ. The IFN-γR2 subunit is a helper receptor subunit, with only a Jak-association site and no Stat-recruitment site. Thus the cytoplasmic domain of any receptor subunit that can bring in a Jak kinase to the IFN-γ receptor complex is functional in supporting signal transduction (See Example 1). Based on the data of Figures 11, 12, and 16, we suggest that the first active kinase that is essential for function of the IFN-γ receptor complex is Jak2, which is recruited by the intracellular domain of the IFN-γR2 chain, and that Jak2 is more effective at phosphorylating Jak1 than the latter is at cross-phosphorylating itself. This is consistent with the data of [Briscoe *et al. J.Interferon and Cytokine Research*, **15**(Supplement 1):S56 (1995)] who reported that a Jak1 molecule with an inactive kinase domain can replace the normal Jak1 in signal transduction by IFN-γ.

The absolute requirement for two distinct Jak kinases in the IFN-γ signalling pathway has been demonstrated by the use of kinase-deficient cell lines [Müller *et al.*, 1993, *supra*; Watling *et al.*, *Nature* **366**:166-170 (1993)]. Based on the results with the chimeric erythropoietin-interferon gamma receptors, we propose that this reflects two features characteristic of the IFN-γ receptor complex: the unique properties of the receptor relative to the positioning of the Jaks, and the

idea that Jak1 is relatively ineffective at transphosphorylation in the inactive state whereas Jak2 is quite effective at activating unphosphorylated Jak1 kinase.

Unlike the growth hormone-receptor complex [De Vos *et al.*, *Science*, 255:306-312 (1992)] and the EpoR dimer [Watowich *et al.*, 1994, *supra*], when one IFN-γ homodimer binds two IFN-γR1 molecules, the two receptor subunits do not interact with one another and are separated by 27Å [Walter *et al.*, 1995, *supra*] at their closest point. Therefore, though the IFN-γR1 chain possesses both a Jak kinase-association site and a Stat-recruitment site, it is alone unable to transduce a signal on homodimerization as the two Jak1 kinases are not in physical proximity to activate each other (Figure 17). The signal-transducing complex of IFN-γ most likely consists of the IFN-γ homodimer bound to two IFN-γR1 and two IFN-γR2 chains (Example 1, *supra*). Binding of IFN-γ to IFN-γR1 facilitates its oligomerization with IFN-γR2, which recruits Jak2 into the receptor complex to initiate signal transduction.

That Jak1 is relatively ineffective alone in transphosphorylation is supported by the observation that cells expressing the EpoR/γR1 chimera are quantitatively less active in the biological assays than are the cells expressing both EpoR/γR1 and EpoR/γR2 chimeric receptor chains. Thus, though homodimerization of the EpoR/γR1 receptor by Epo brings the cytoplasmic domains of the two γR1 subunits into close enough proximity to transduce a signal on homodimerization, the two Jak1 kinases do not transactivate each other as efficiently as they are activated by Jak2. It should, however, also be considered that the physical conformation of the intracellular domains of the EpoR/γR1 chimeric receptor homodimer may be different from that of the intracellular domains of the native IFN-γR1 receptor homodimers, permitting a low-level activation of the two adjoining Jak1 kinase molecules.

In the case of the EpoR/γR1 - EpoR/γR2 dimer, one Jak1 and one Jak2 kinase are in close apposition for Jak2 to phosphorylate Jak1 and set in motion efficient

downstream signaling events. That Jak1 plays an important structural role in the IFN-γ receptor complex is suggested by the observation that cells expressing the EpoR/γR2(p91) chimeric receptor subunit are muted in their biological responses compared to cells expressing both EpoR/γR1 and EpoR/γR2.

In cells expressing both EpoR/γR1 and EpoR/γ2 receptor chains, binding of the ligand Epo can induce the formation of three types of receptor dimers: EpoR/γR1 homodimers, EpoR/γ2 homodimers and EpoR/γR1-EpoR/γR2 heterodimers. The EpoR/γR1 homodimer is only weakly active and the EpoR/γR2 homodimer is inactive. The functional receptor complex therefore must be the EpoR/γR1-EpoR/γR2 hetero-oligomer, with the two receptor subunits in a 1:1 ratio. It remains to be conclusively demonstrated, however, if the active chimeric EpoR receptor complex is a heterodimer or a heterotetramer. In the former case the phosphorylated Stat1α from each receptor dimer would be released into the cytoplasm where it dimerizes. In the latter case two phosphorylated Stat1α molecules in each tetrameric receptor complex would dimerize and then be released from the receptor complex. The EpoR is only known to form a dimer; however, in all receptor complexes that activate Stats thus far studied, at least two Stat molecules per receptor complex are recruited. Therefore it is thought that the phosphorylated Stats dimerize on the receptor complex where they are in close proximity and are subsequently released. The activation of Stat 1α, then, indirectly supports a conclusion that the EpoR/γR2 chimeric receptors form a heterotetrameric complex.

A model for the signal transduction of the IFN-γR complex is shown in Figure 17, which illustrates schematically the structures of the various receptor complexes. The IFN-γ homodimer binds to two IFN-γR1 chains, followed by its interaction with two IFN-γR2 chains. Although Jak1 binds to the intracellular domain of IFN-γR1, a functional kinase activity on Jak1 is probably not required for signal transduction [Briscoe *et al.*, 1995, *supra*]. Thus the first active kinase that is essential for function of the IFN-γR complex is Jak2 which is recruited by the

intracellular domain of the IFN-γR2 chain. When the receptor oligomerizes, two Jak2 kinases per receptor complex are incorporated into the active complex. We suggest that Jak2 phosphorylates Jak1, following which either kinase phosphorylates tyrosine 457 of the IFN-γR1 chain. Phosphorylation of the IFN-γR1 chain is an absolute requirement for its function [Cook *et al.*, 1992, *supra*; Farrar *et al.*, *Proc. Natl. Acad. Sci. USA.* **89**:11706-11710 (1992); Greenlund *et al.*, 1994, *supra*]. The phosphorylated segment of each IFN-γR1 chain then recruits unphosphorylated Stat1α to the complex, which is then phosphorylated by Jak2 or Jak1 kinase. The phosphorylation of the Stat1α molecules causes their release from the receptor complex and their subsequent dimerization to form the active Stat1α transcription unit, which translocates into the nucleus. Jak2 and the associated IFN-γR2 chain thus play a pivotal role in the signal transduction process.

The highly species-specific interaction of the extracellular domain of the IFN-γR2 chain with the extracellular domain of the IFN-γR1 chain-IFN-γ ligand complex provides for specific signal transduction established by IFN-γ [Jung *et al.*, 1987, *supra*; Hibino *et al.*, 1992, *supra*; Hemmi *et al.*, 1992, *supra*; Gibbs *et al.*, (1991) *supra*]. In normal cells, other receptor components that can recruit Jak2 to their respective complexes cannot substitute for the specific IFN-γR2 chain unless they have the specific IFN-γR2 chain extracellular domain.

The Jak kinases do not mediate ligand selectivity and seem to be promiscuous in their activity as shown in Example 1, which demonstrates that each of the Jak kinases can substitute for Jak2 in signal transduction by IFN-γ. Selectivity is likely maintained from the extracellular receptor-ligand interaction to the final signal transduction mechanism by other specific regions of the intracellular domains. For example, Heim *et al.* *Science*, **267**:1347-1349 (1995) suggested that the SH2 recognition domain of Stat1α maintains some of the specificity. It remains to be established, however, how Stat1α can be activated by many different cytokines and their receptors and maintain specificity through the final

transcription activation step. Our current data delineate the role of the intracellular domain of the IFN-γR2 chain in the IFN-γ signal transduction complex. Furthermore, it is possible that the simultaneous recruitment of Jak2 by other cytokines to their own receptors on the cell surface increasing the subcytoplasmic membrane concentration of Jak2 may explain some of the cross talk among the biochemical mechanisms of cytokines and growth factors.

Finally, but not unimportantly, the present Example clearly demonstrates the unexpected ability to reconstitute a highly complex signal transduction pathway involving a heterotetrameric receptor complex with chimeric receptors.

EXAMPLE 4: A Functional Immunoglobulin/Interferon-γ Chimeric Receptor

A synthetic receptor to a tumor-associated antigen TAG72 was prepared, using the heavy chain antigen binding domain joined to IFN-γR1, and the light chain antigen binding domain joined to IFN-γR2. Cells transfected with plasmids encoding for the chimeric receptors were activated when exposed to fixed (antigenic) cells carrying the tumor-associated antigen TAG72.

Materials and Methods

Chimeric constructs. To construct chimera L1/R2 the PCR reaction was performed with 5'-ATTCGGATCCAGGATGGATTC-3' (SEQ ID NO:35) and 5'-CGGCGCTAGCCACTGCTGTTCCAGCACCAGCTTGGTC-3' (SEQ ID NO:36) primers with light chain gene of HuCC49 as a template [Kashmiri *et al.*, *Hybridoma* 14:461-473 (1995)]. A *Bam*HI or *Nhe*I restriction site was present in the oligonucleotides above, respectively. The PCR product was digested with *Nhe*I and *Bam*HI restriction endonucleases and ligated into the *Nhe*I and *Bam*HI sites of the plasmid pR2/R2. The resultant plasmid was designated pL1/R2. The aminoglycoside phosphotransferase gene (this gene encodes the protein rendering mammalian cells resistant to antibiotic G418) was disrupted by digestion of the pL1/R2 plasmid with *Nar*I and *Sfu*I restriction endonucleases and religation of the plasmid DNA.

To construct chimera H1/R1, the PCR reaction was performed with 5'-CCAGTGGATCCCCCGGGCTG-3' (SEQ ID NO:37) and 5'-CGGCGCTAGCCATTTGGCTGAGGAGACGGTG-3' (SEQ ID NO:38) primers and the heavy chain gene of HuCC49 as a template [Kashmiri *et al.*, *supra*] with the *Bam*HI and *Nhe*I restriction sites present in the oligonucleotides above, respectively. The PCR product was digested with *Nhe*I and *Bam*HI restriction endonucleases and ligated into the *Nhe*I and *Bam*HI sites of the plasmid pR2/R1. The resultant plasmid was designated pH1/R1. The *neo* gene that conferred resistance to antibiotic G418 was retained on this plasmid.

Cells were stably cotransfected with the expression vectors (1-3 g of super-coiled plasmid DNA with the neo^R gene and a 10-fold excess of plasmid DNA without neo^R gene per 10^5-10^6 cells) with LipofectAMINE™ Reagent (Life Technologies, Inc.) according to the manufacturer's instructions for stable transfection of adherent and suspension cells. All cell lines transfected with plasmids carrying the neo^R gene were selected and maintained in medium containing 450 μg/ml of antibiotic G418. Complete F12 medium with 10% FBS was used for 16-9 cells; and complete RPMI 1640 medium supplemented with 10% FBS and 10% of conditioned medium from NIH3T3 cells expressing IL-2 for CTL1-2 cells.

Results and Discussion

16-19 cells were co-transfected with two expression plasmids encoding chimeric antibody-receptor molecules L1/γR2 (V_L domain of light chain of the cc49 antibody fused to the transmembrane and intracellular domains of human IFN-γR2 chain) and H1/γR1 (V_L domain of heavy chain of cc49 antibody fused to the transmembrane and intracellular domains of human IFN-γR1 chain). These cells, and untransfected parental 16-9 controls, were treated or left untreated with paraformadehyde fixed MCF-7 clone 4C10 cells for 72 hours. MCF-7 cells express the TAG72 antigen. HLA-B7 antigen expression was detected by treatment of cells with mouse anti-HLA monoclonal antibody W6/32, followed by treatment with FITC-conjugated goat anti-mouse IgG. As shown in Figure 18, co-

transfected cells that expressed H1/γR1-L1/γR2 responded to the antigen, whereas control cells and unchallenged cells did not.

The two expression plasmids encoding chimeric antibody-receptor molecules L1/γR2 and H1/γR1 were also co-transfected into mouse CTL1-2 cells. Expression of the chimeric antibodies on the cell surface of 16-9 and CTL1-2 cells was detected by treatment of cells with FITC-conjugated goat anti-mouse IgG. The cells were then analyzed by cytofluorography. As shown in Figure 19, both 16-9 and CTL1-2 cells co-transfected with the L1/γR2 and H1/γR1 expression vectors have detectable antibody on their surface, whereas un-co-transfected controls lack antibody.

These data show that a chimeric antibody/IFN-γ receptor complex is able to generate the signal transduction event analogous to that procedure by interferon gamm (IFNγ). Signal transduction was stimulated by interacting the synthetic receptor carrying the variable region of the antibody that reacts with the tumor-associated antigen TAG72 that is expressed on the breast carcinoma cell line MCF07 (Figure 18). Synthetic receptor molecules carrying heavy and light chain domains are expressed in both the 16-9 and CTL1-2 cells (Figure 19).

The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A chimeric receptor for cellular activation comprising an extracellular domain capable of binding to a cognate molecule, and a cytokine intracellular domain capable of interacting with a Jak-Stat protein, thereby participating in signal transduction, with the proviso that the cytokine intracellular domain is not naturally associated with the extracellular domain.

2. The chimeric receptor of claim 1 wherein the cytokine intracellular domain interacts with a Jak protein selected from the group consisting of Jak1, Jak2, Jak3, and Tyk2.

3. The chimeric receptor of claim 1 wherein the cytokine intracellular domain interacts with a Stat protein selected from the group consisting of Stat1α, Stat1β, Stat2, Stat3, Stat4, Stat5, and Stat6.

4. The chimeric receptor of claim 1 wherein the extracellular domain is selected from the group consisting of a cytokine and a growth factor.

5. The chimeric receptor of claim 4 wherein the cytokine is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-10, interleukin-12, and erythropoietin.

6. The chimeric receptor of claim 1 wherein the extracellular domain is a receptor ligand binding extracellular domain.

7. The chimeric receptor of claim 6 wherein the receptor ligand binding extracellular domain is selected from the group consisting of an antibody antigen binding domain, a cytokine receptor ligand binding domain, and a growth factor receptor ligand binding domain.

8. The chimeric receptor of claim 7 wherein the cytokine receptor ligand binding domain is an extracellular domain selected from the group consisting of Interferon-α, Interferon-β, Interferon-γ, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-10, interleukin-12, and erythropoietin.

9. The chimeric receptor according to claim 7 wherein the antibody antigen binding domain is selected from the group consisting of a heavy chain extracellular domain, a heavy chain-light chain complex extracellular domain, and a single chain F_v domain.

10. A nucleic acid encoding a chimeric receptor for cellular activation comprising an extracellular domain capable of binding to a cognate molecule, and a cytokine intracellular domain capable of interacting with a Jak-Stat protein, thereby participating in signal transduction, with the proviso that the cytokine intracellular domain is not naturally associated with the extracellular domain.

11. The nucleic acid encoding a chimeric receptor of claim 10 wherein the cytokine intracellular domain interacts with a Jak protein selected from the group consisting of Jak1, Jak2, Jak3, and Tyk2.

12. The nucleic acid encoding a chimeric receptor of claim 10 wherein the cytokine intracellular domain interacts with a Stat protein selected from the group consisting of Stat1α, Stat1β, Stat2, Stat3, Stat4, Stat5, and Stat6.

13. The nucleic acid encoding a chimeric receptor of claim 10 wherein the extracellular domain is selected from the group consisting of a cytokine and a growth factor.

14. The nucleic acid encoding a chimeric receptor of claim 13 wherein the cytokine is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-10, and interleukin-12.

15. The nucleic acid encoding a chimeric receptor of claim 10 wherein the extracellular domain is a receptor ligand binding extracellular domain.

16. The nucleic acid encoding a chimeric receptor of claim 15 wherein the receptor ligand binding extracellular domain is selected from the group consisting of an antibody antigen binding domain, a cytokine receptor ligand binding domain, and a growth factor receptor ligand binding domain.

17. The nucleic acid encoding a chimeric receptor of claim 16 wherein the cytokine receptor ligand binding domain is an extracellular domain selected from the group consisting of Interferon-α, Interferon-β, Interferon-γ, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-8, interleukin-10, and interleukin-12.

18. The nucleic acid encoding a chimeric receptor according to claim 16 wherein the antibody antigen binding domain is selected from the group consisting of a heavy chain extracellular domain, a heavy chain-light chain complex extracellular domain, and a single chain F_v domain.

19. An expression vector comprising the nucleic acid of claim 10 operably associated with an expression control sequence.

20. A genetically engineered host cell comprising the expression vector of claim 19.

21. A method for reconstituting responsiveness to a soluble factor comprising administering the expression vector of claim 19 to cells of a host animal lacking responsiveness to a soluble factor, wherein the chimeric receptor encoded by the expression vector comprises an extracellular domain that binds to the soluble factor.

22. Targeted effector cells comprising an expression vector of claim 19, wherein the chimeric receptor encoded by the expression vector comprises an extracellular domain which is an antibody antigen binding domain.

23. The targeted effector cells of claim 22, wherein the effector cells are selected from the group consisting of natural killer cells, lymphokine activated killer cells, cytotoxic T cells, macrophages/monocytes, neutrophils, basophils, and polymorphonuclear leukocytes.

24. A method for treating a disease or disorder characterized by disregulation of a cellular activity comprising administering the effector cells of claim 22 targeted to the cell which is undergoing disregulation.

25. The method according to claim 24 wherein the disease or disorder is selected from the group consisting of viral infection, cancer, inflammatory disease, and autoimmune disease.

26. Targeted effector cells comprising an expression vector of claim 19, wherein the chimeric receptor encoded by the expression vector comprises an extracellular domain which is selected from the group consisting of a cytokine and a growth factor.

27. The targeted effector cells of claim 26, wherein the effector cells are selected from the group consisting of natural killer cells, lymphokine activated killer cells, cytotoxic T cells, macrophages/monocytes, neutrophils, basophils, and polymorphonuclear leukocytes.

28. A method for treating a disease or disorder characterized by disregulation of a cellular activity comprising administering the effector cells of claim 26 targeted to the cell which is undergoing disregulation.

29. The method according to claim 28 wherein the disease or disorder is selected from the group consisting of viral infection, cancer, inflammatory disease, and autoimmune disease.

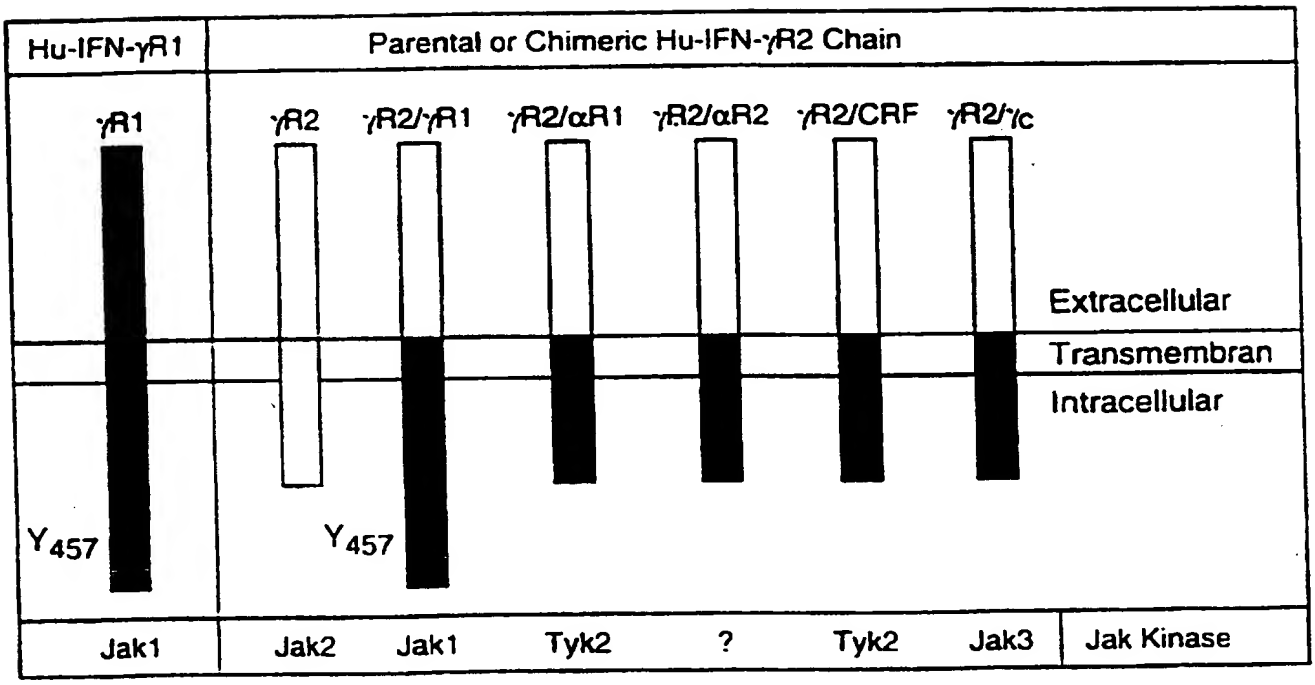

Hu-IFN-γR1
Parental or Chimeric Hu-IFN-γR2 Chain
γR1
γR2
γR2/γR1
γR2/αR1
γR2/αR2
γR2/CRF
γR2/γc
Extracellular
Transmembran
Intracellular
Y457
Y457
Jak1
Jak2
Jak1
Tyk2
?
Tyk2
Jak3
Jak Kinase

FIG. 1

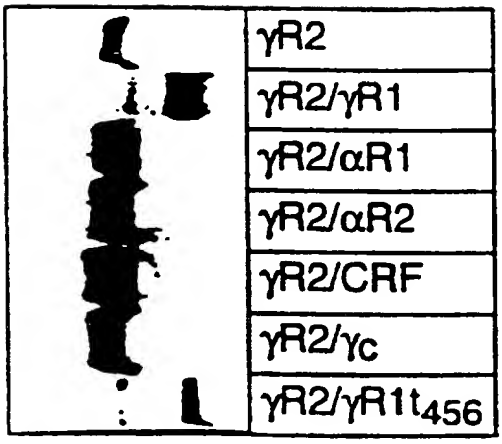
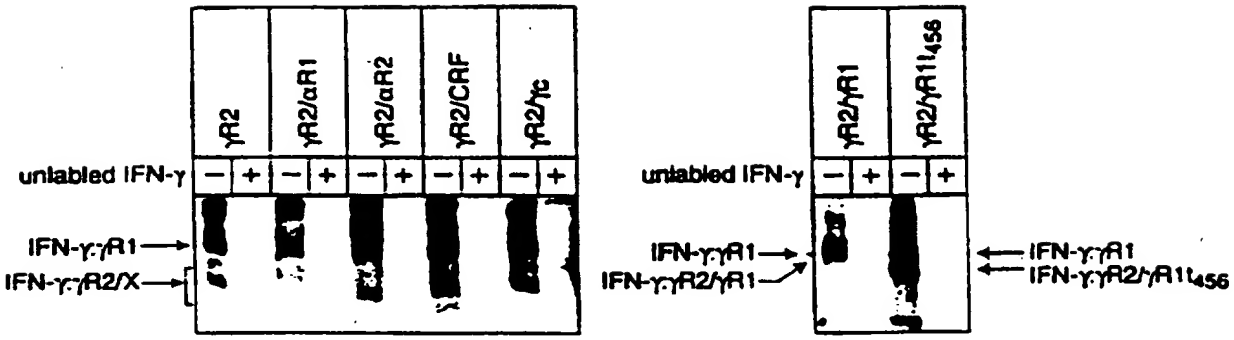


FIG. 2


γR2
γR2/αR1
γR2/αR2
γR2/CRF
γR2/γc
unlabled IFN-γ
IFN-γ:γR1
IFN-γ:γR2/X
γR2/γR1
γR2/γR1l456
unlabled IFN-γ
IFN-γ:γR1
IFN-γ:γR2/γR1
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IFN-γ:γR2/γR1l456

FIG. 3

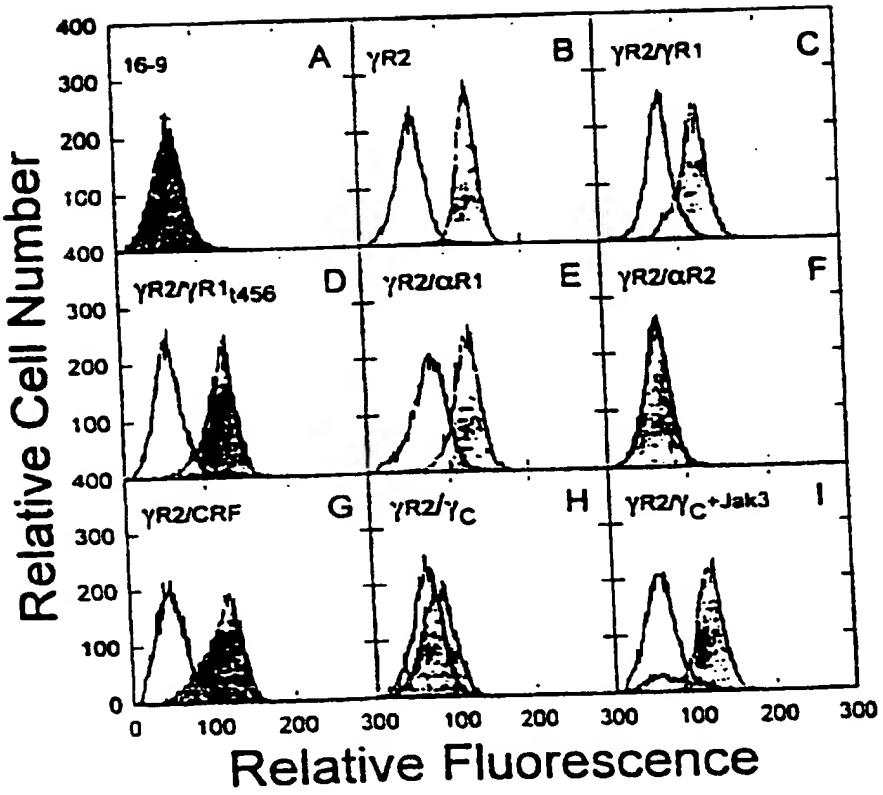
16-9
A
γR2
B
γR2/γR1
C
γR2/γR1t456
D
γR2/αR1
E
γR2/αR2
F
γR2/CRF
G
γR2/γC
H
γR2/γC+Jak3
I
Relative Cell Number
Relative Fluorescence
0
100
200
300
400

FIG. 4

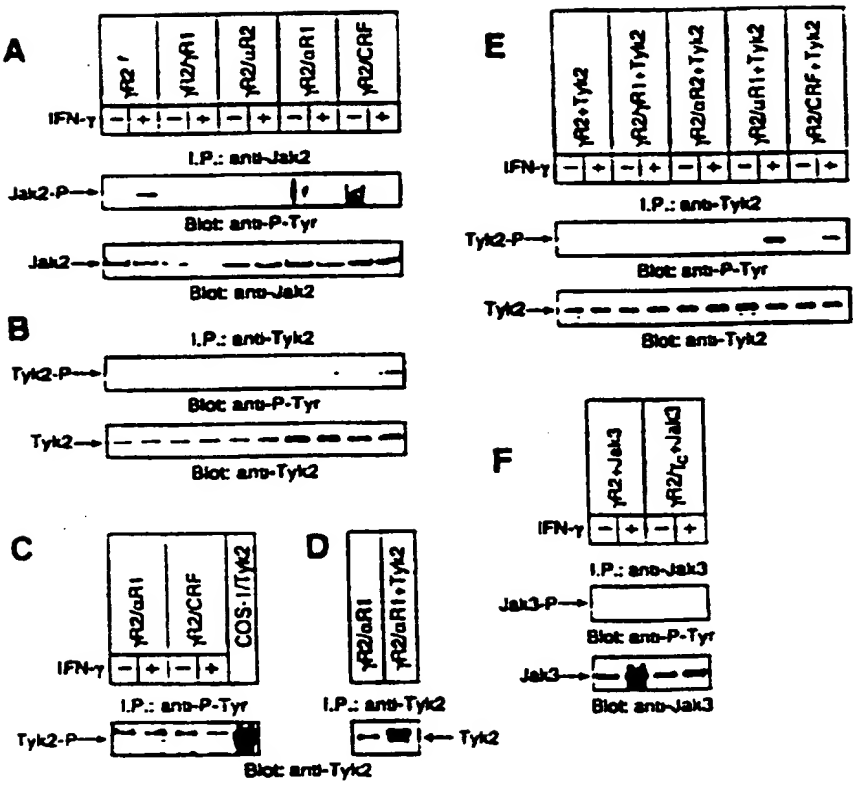
A
γR2'
γ12/γR1
γR2/αR2
γR2/αR1
γR2/CRF
IFN-γ
I.P.: anti-Jak2
Jak2-P
Blot: anti-P-Tyr
Jak2
Blot: anti-Jak2
B
I.P.: anti-Tyk2
Tyk2-P
Blot: anti-P-Tyr
Tyk2
Blot: anti-Tyk2
C
γR2/αR1
γR2/CRF
COS-1/Tyk2
IFN-γ
I.P.: anti-P-Tyr
Tyk2-P
Blot: anti-Tyk2
D
γR2/αR1
γR2/αR1+Tyk2
I.P.: anti-Tyk2
Tyk2
E
γR2+Tyk2
γR2/γR1+Tyk2
γR2/αR2+Tyk2
γR2/αR1+Tyk2
γR2/CRF+Tyk2
IFN-γ
I.P.: anti-Tyk2
Tyk2-P
Blot: anti-P-Tyr
Tyk2
Blot: anti-Tyk2
F
γR2+Jak3
γR2/γc+Jak3
IFN-γ
I.P.: anti-Jak3
Jak3-P
Blot: anti-P-Tyr
Jak3
Blot: anti-Jak3

FIG. 5

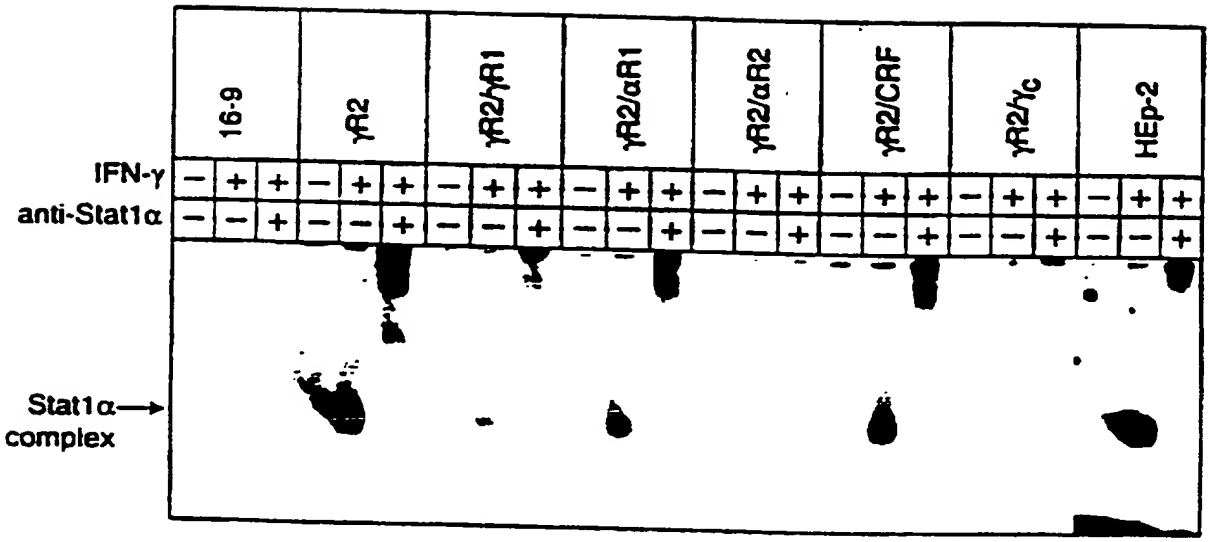
16-9
γR2
γR2/γR1
γR2/αR1
γR2/αR2
γR2/CRF
γR2/γc
HEp-2
IFN-γ
anti-Stat1α
Stat1α
complex

FIG. 6

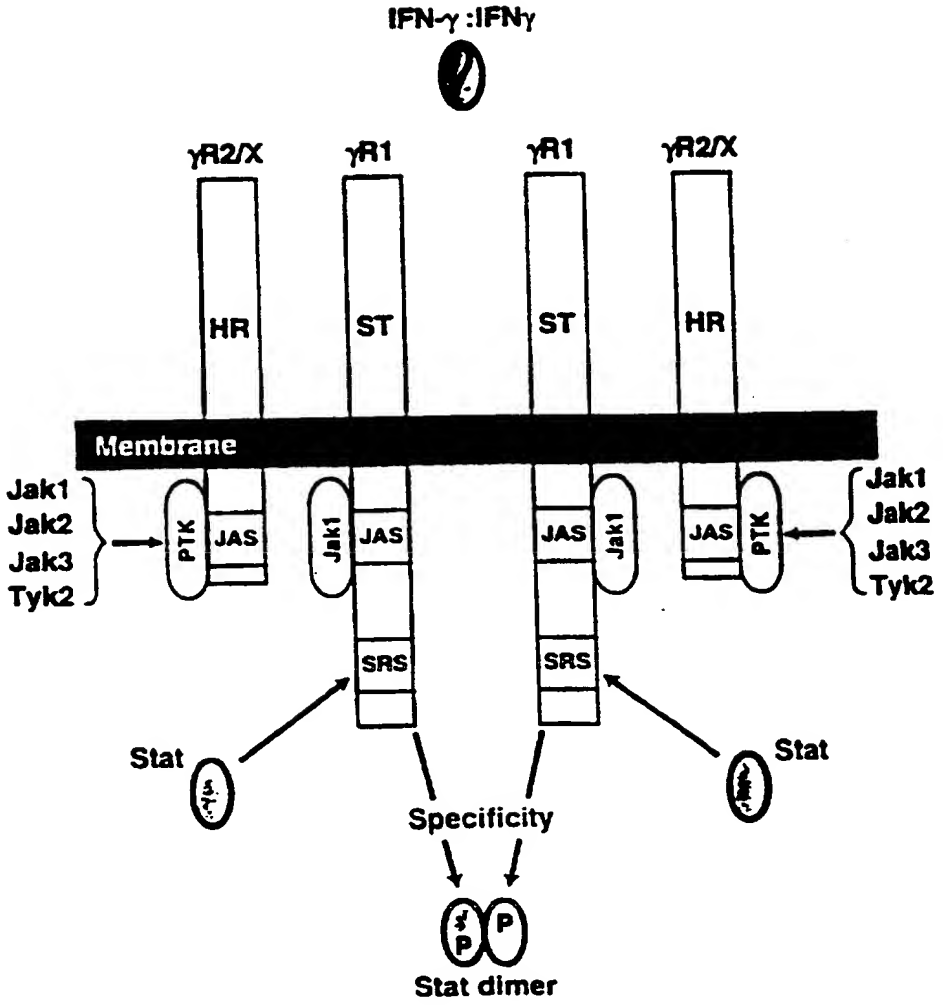
IFN-γ :IFNγ
γR2/X
γR1
γR1
γR2/X
HR
ST
ST
HR
Membrane
Jak1
Jak2
Jak3
Tyk2
PTK
JAS
Jak1
JAS
JAS
Jak1
JAS
PTK
Jak1
Jak2
Jak3
Tyk2
SRS
SRS
Stat
Stat
Specificity
P
P
Stat dimer

FIG. 7

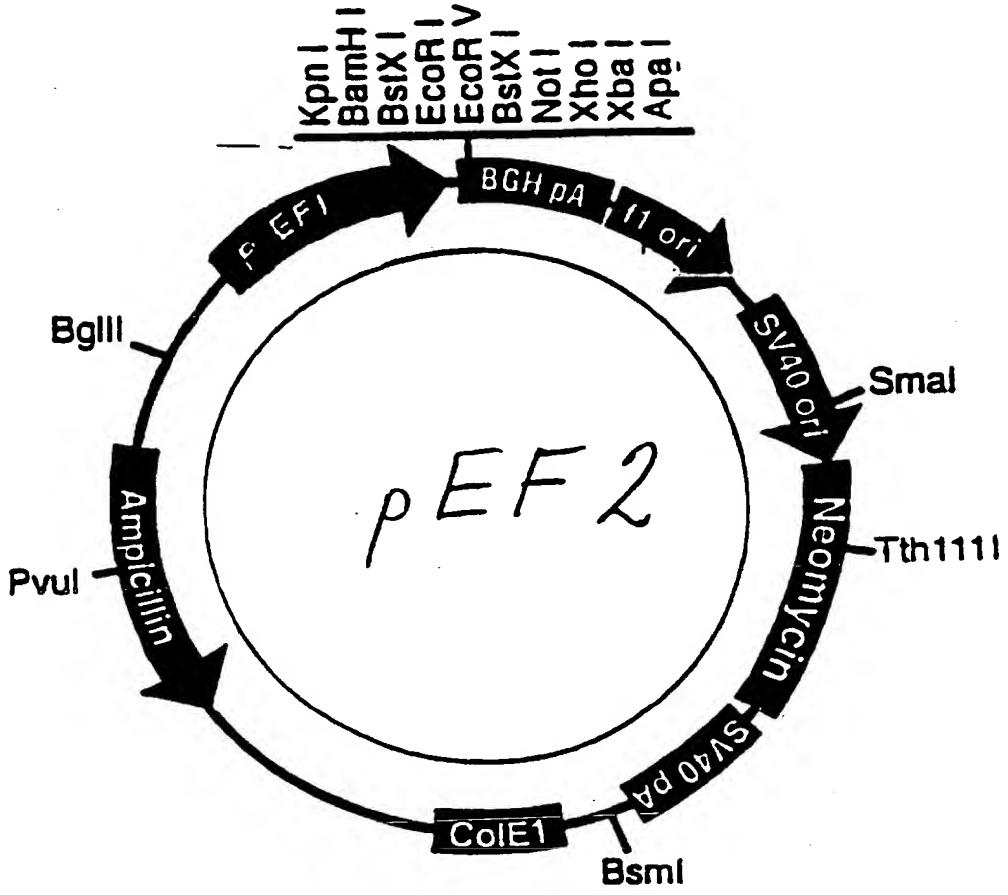
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
BGH pA
f1 ori
SV40 ori
SmaI
Tth111I
Neomycin
SV40 pA
ColE1
BsmI
Ampicillin
PvuI
BglII
pEF2

FIG. 8

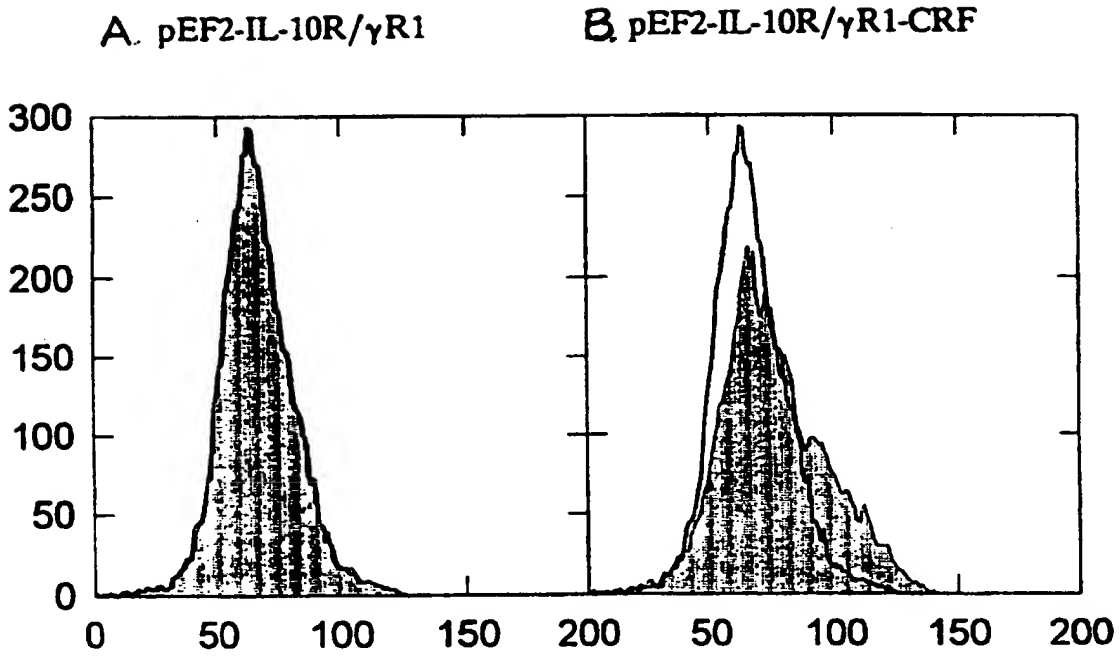
A. pEF2-IL-10R/γR1
B. pEF2-IL-10R/γR1-CRF
300
250
200
150
100
50
0
0
50
100
150
200
50
100
150
200

FIG. 9

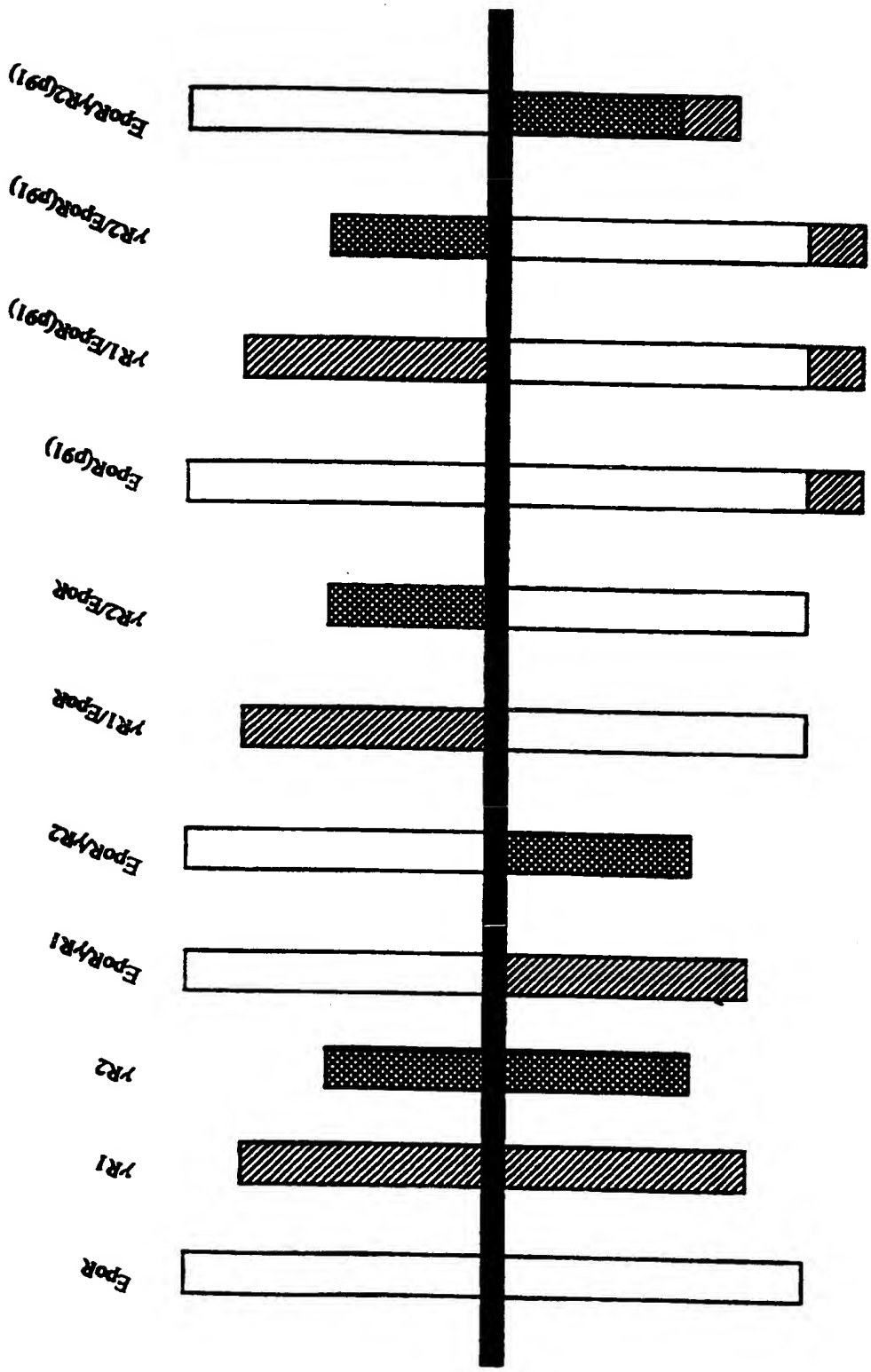

FIG. 10

Jak	Jak2	Jak1	Jak2	Jak1	Jak2	Jak2	Jak2	Jak2	Jak2
Stat	Stat5	Stat1α	-	Stat1α	-	Stat5	Stat5	Stat5 Stat1α	Stat5 Stat1α	Stat5 Stat1α	Stat1α

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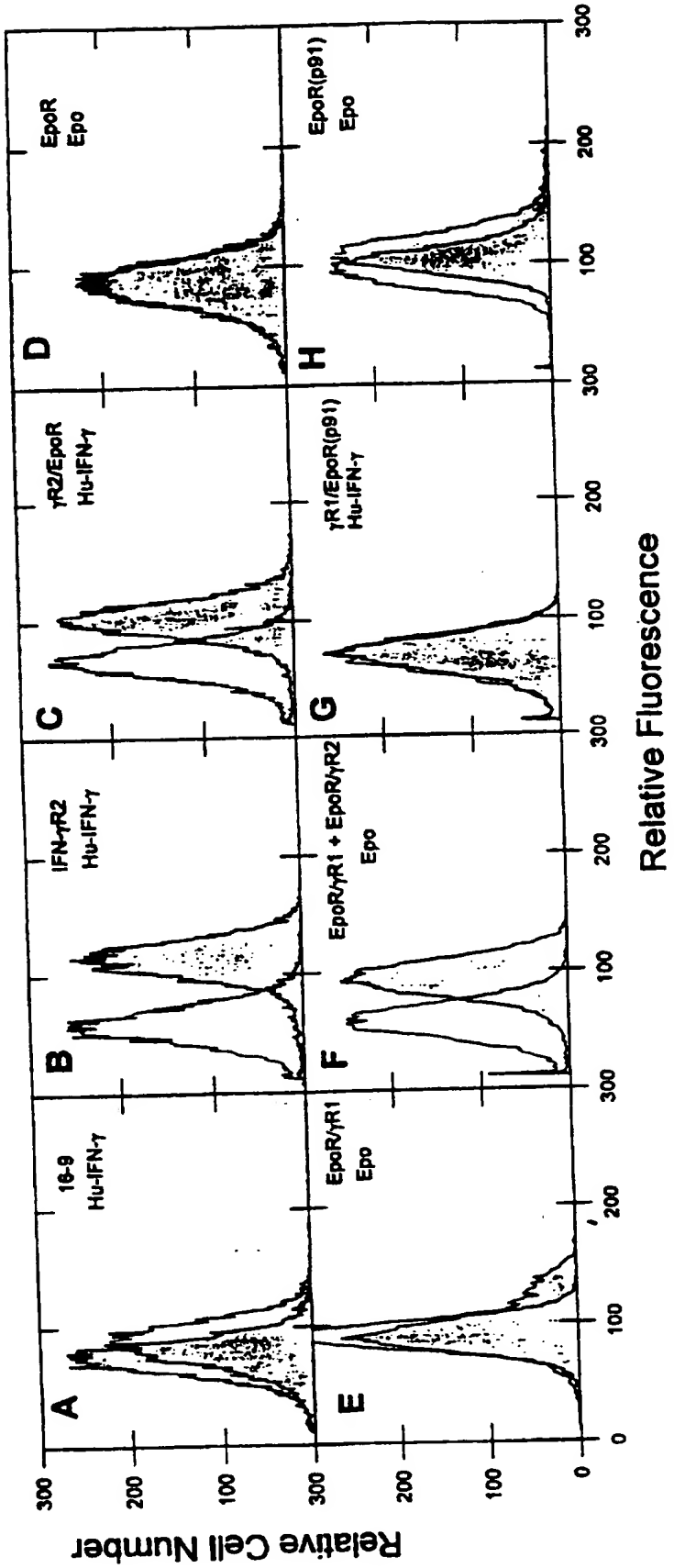

FIG. 11

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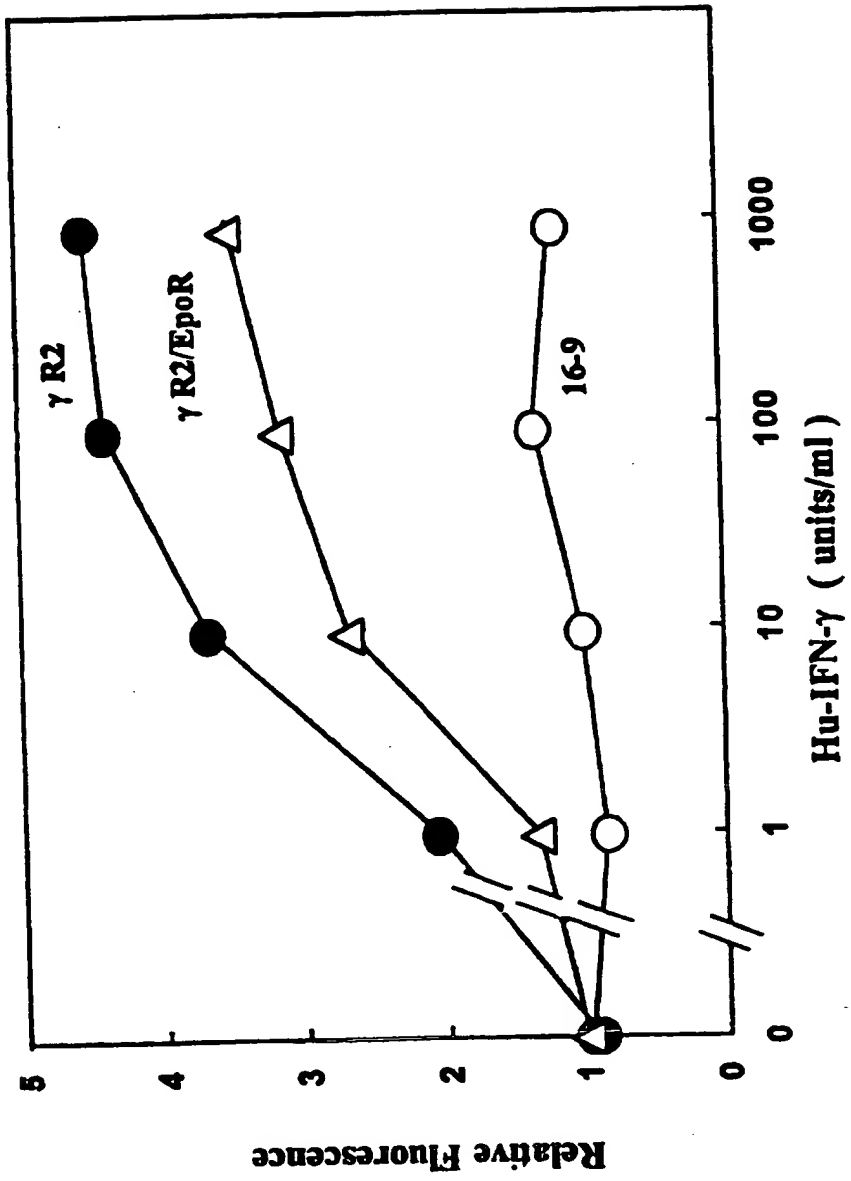

FIG. 12

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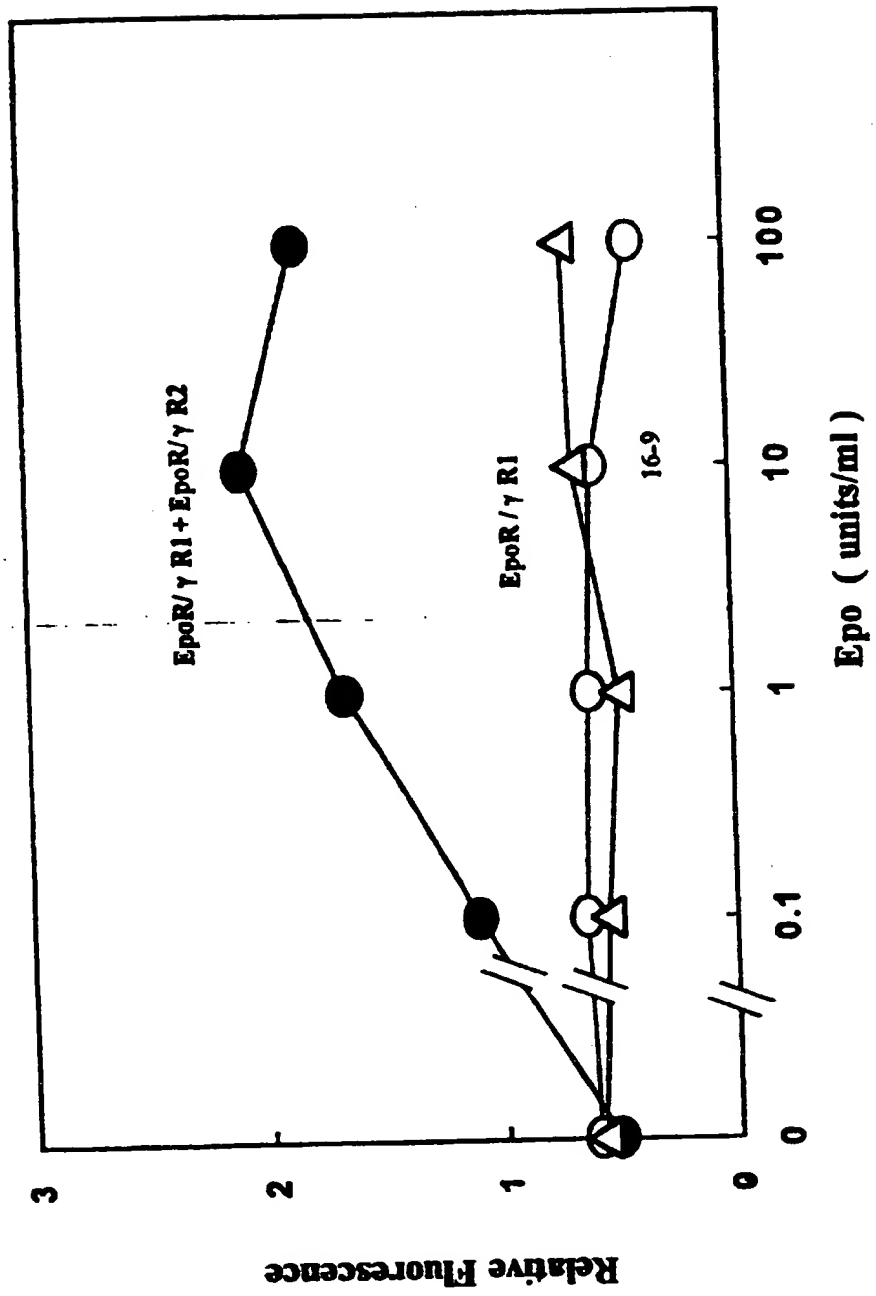

FIG. 13

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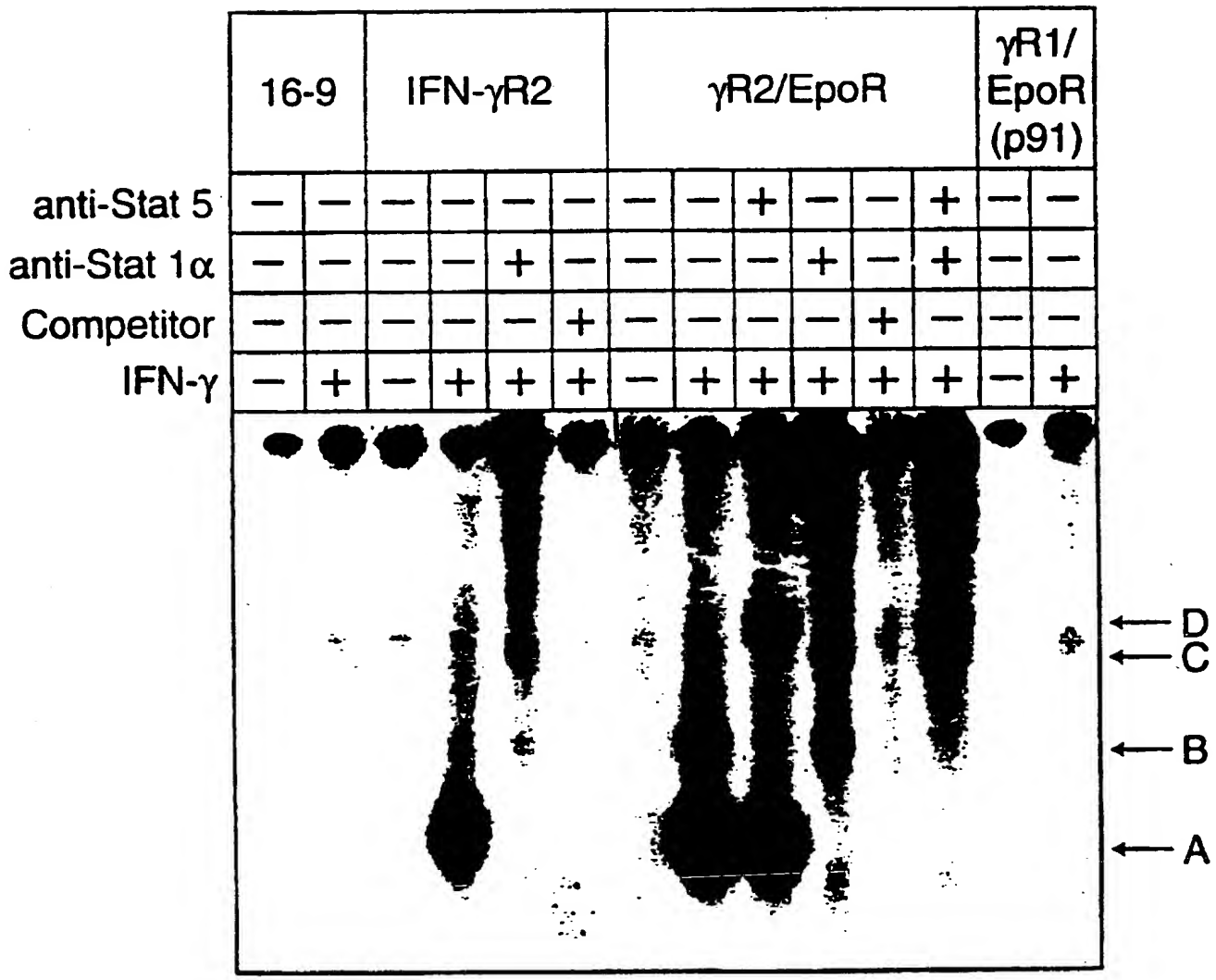
16-9
IFN-γR2
γR2/EpoR
γR1/ EpoR (p91)
anti-Stat 5
anti-Stat 1α
Competitor
IFN-γ
D
C
B
A

FIG. 14

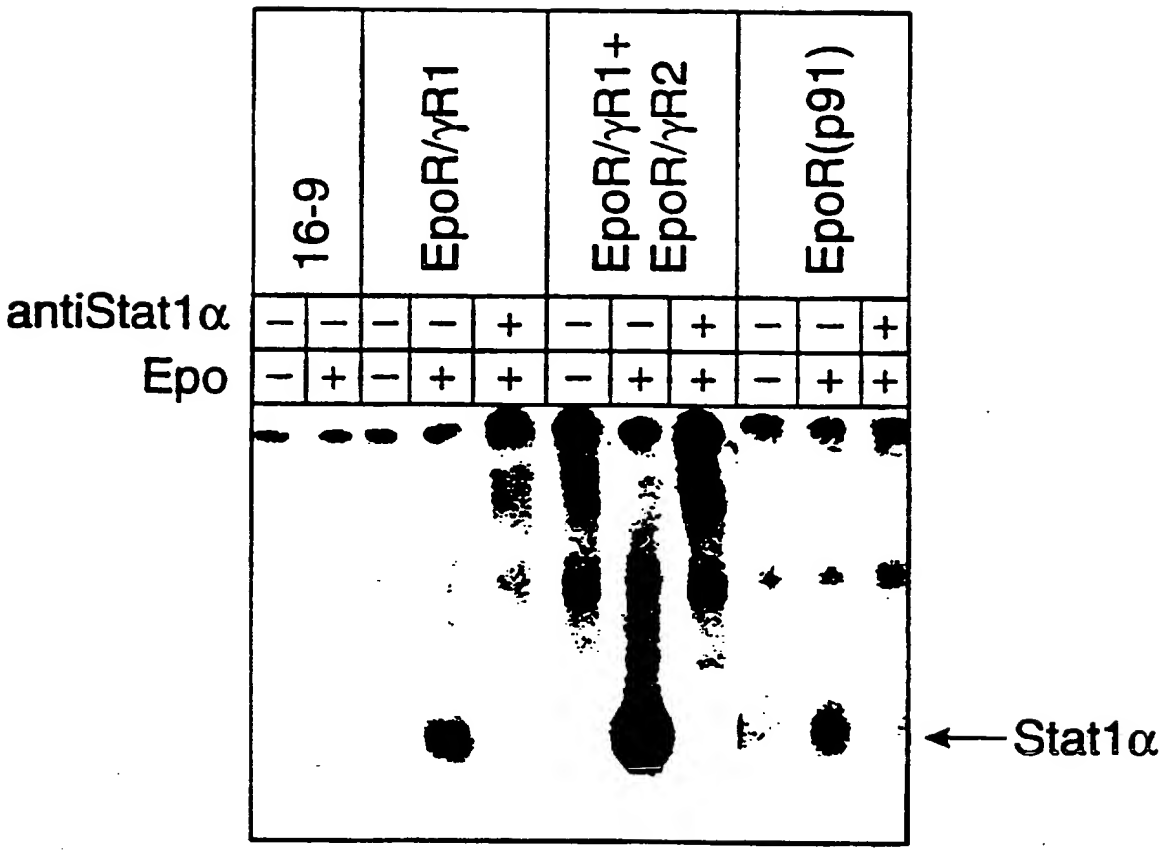
16-9
EpoR/γR1
EpoR/γR1+
EpoR/γR2
EpoR(p91)
antiStat1α
Epo
– – – – + – – + – – +
– + – + + – + + – + +
Stat1α

FIG. 15

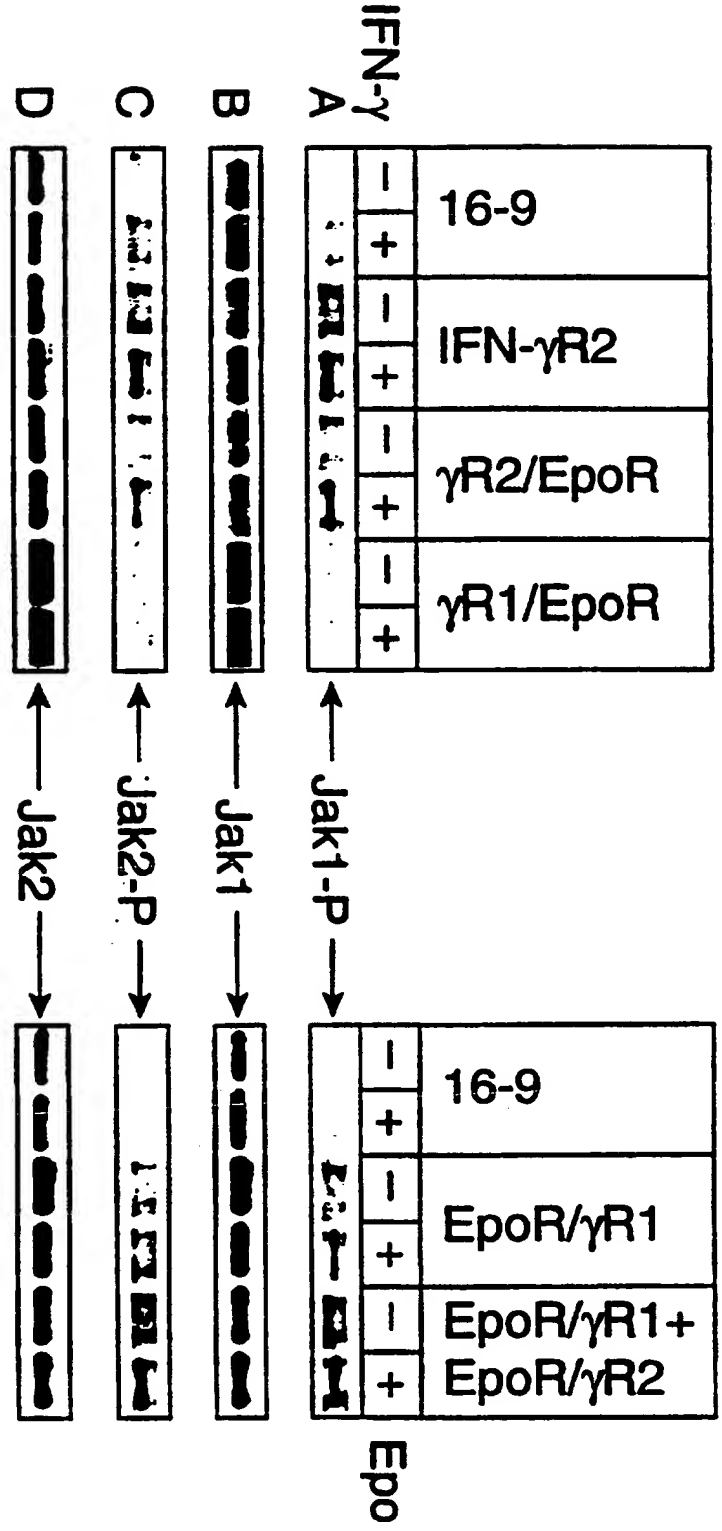
IFN-γ
A
B
C
D
16-9
IFN-γR2
γR2/EpoR
γR1/EpoR
Jak1-P
Jak1
Jak2-P
Jak2
16-9
EpoR/γR1
EpoR/γR1+
EpoR/γR2
Epo

FIG. 16

601/1/043 (Sheet 17 of 19)

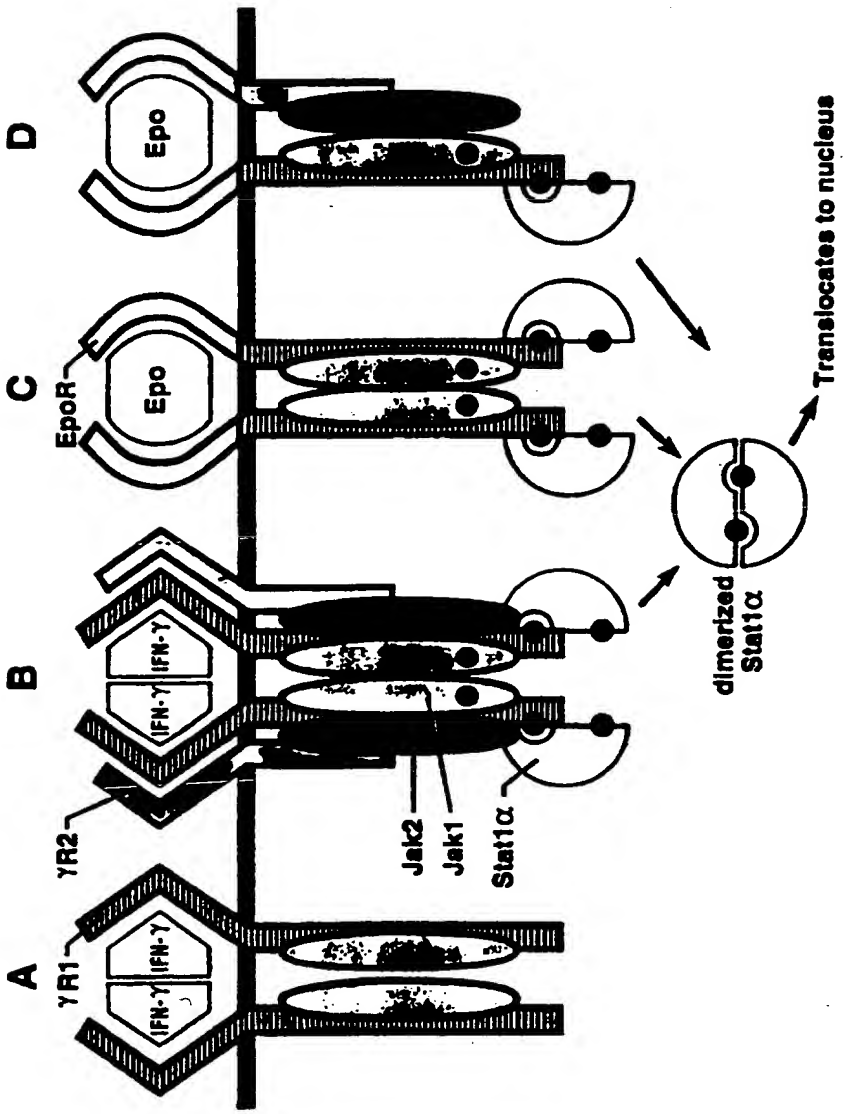

FIG. 17

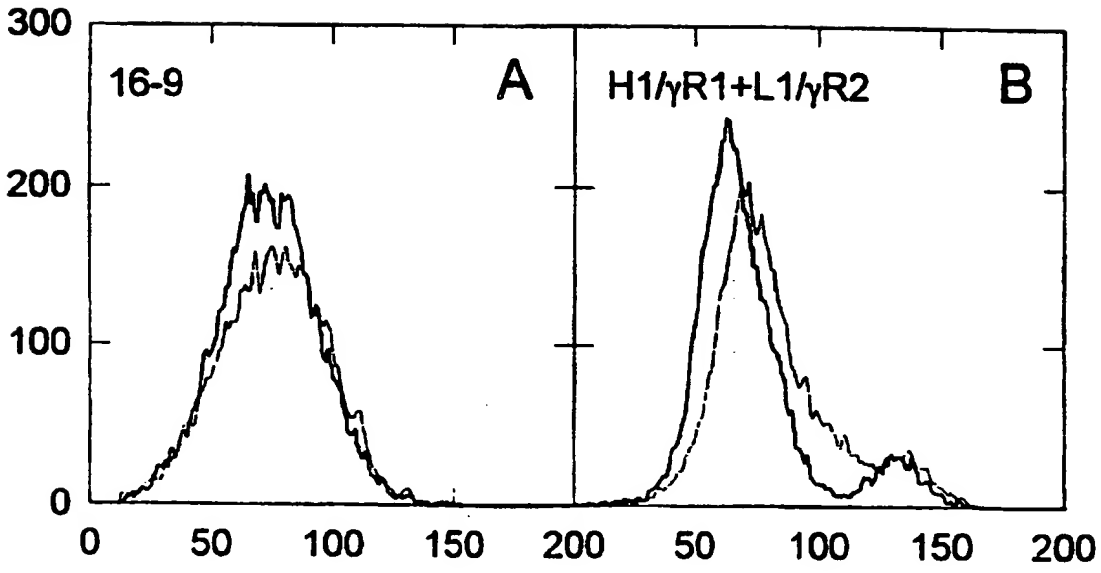

FIG. 18

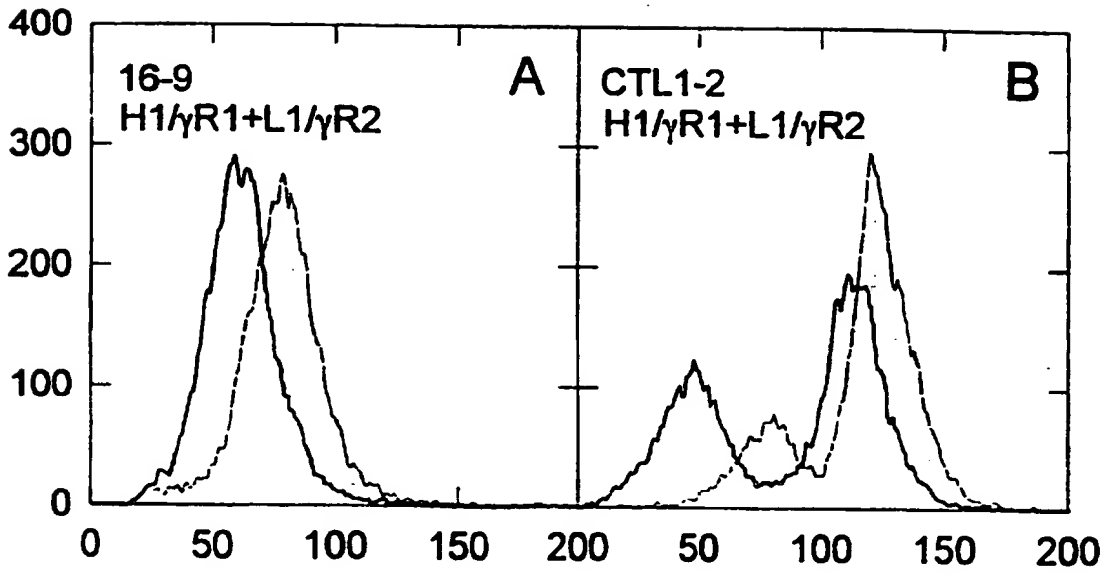
400
300
200
100
0
16-9
H1/γR1+L1/γR2
A
CTL1-2
H1/γR1+L1/γR2
B
0
50
100
150
200
50
100
150
200

FIG. 19